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Do competitive conditions affect introgression of transgenes from oilseed rape (*Brassica napus*) to weedy *Brassica rapa*?

-A case study with special reference to transplastomic oilseed rape

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Abstract (max. 2000 char.):
In species where chloroplast inheritance is exclusively or predominantly maternal, pollen-mediated flow of transgenes is reduced if transgenes are inserted in chloroplast DNA instead of nuclear DNA. However, transmission of chloroplast-encoded transgenes will still occur if transgenic individuals act as the maternal parent when hybridisation and backcrossing takes place.
Chloroplast DNA inheritance between F_1-hybrids (*B. napus* (♀) x *B. rapa*) and *B. rapa*; the second step in the introgression process of transgenes from transplastomic *B. napus* to *B. rapa* was investigated. It was maternal in all 122 examined cases.
Field trials with *B. napus* and *B. rapa* coexisting in different proportions and densities elucidated how these factors affect the F_1-hybrid production on *B. napus*. Higher plant density reduced the fitness of mother plants and the abundance of F_1-hybrids (at the 1:1 proportion) significantly. As to the proportion between the species, *B. rapa* was a stronger competitor than *B. napus*. The proportion seemed to be a more powerful factor than the density. In conclusion, hybridisation on *B. napus* seems to be most likely at current field densities of *B. napus* and when *B. rapa* is an abundant weed.
The next step in the introgression process was investigated in field trials with F_1-hybrids coexisting with *B. napus* and *B. rapa* in different proportions and densities. With the highest abundance of F_1-hybrids, *B. napus* was the predominant father and the sirering success of the three possible fathers depended on the density. Progenies from F_1-hybrid mother plants grown at the other two proportions were screened merely for individuals sired by *B. rapa* (BC_{1r}s). The density affected on the production of BC_{1r}s significantly but the effect differed among proportions with both the highest and lowest frequencies of BC_{1r}s obtained at high plant density. With low abundance of *B. rapa* the numbers of $BC_{1r}s/m^2$ were low and with high abundance of F_1-hybrids it was comparatively high.
The fitness of mother plants (F_1-hybrids) decreased significantly from low to intermediate density. A further increase only affected the thousand-kernel weight significantly. It was concluded that further introgression of transgenes from transplastomic oilseed rape to *B. rapa* is most likely at current field densities of *B. napus* and when *B. rapa* is an abundant weed.

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Preface

This thesis is submitted to the Institute of Biology, University of Copenhagen as the written part required for the Ph.D. degree.

Since I began my Ph.D. in June 1998, many things have happened. I have had the great pleasure to become the mother of two girls, however the youngest was born with multiple handicaps, which was and is challenging in many aspects. It necessitated a prolonged leave as well as part time employment during a little more than a year.

My Ph.D. was further paused by a three months leave where I worked as research assistant on a project concerning CO_2 exploitation in different varieties of oat.

During the Ph.D. study, I worked three months in the Biotechnology group, Danish Institute of Agricultural Sciences situated at the Royal Veterinary and Agricultural University, with the purpose to acquire experience with another research environment. Senior scientist Merete Albrechtsen was my supervisor and I want to thank her for her involvement and competent supervision, as well as her kindness.

In the Ecological Risk Assessment Group at Risø National Laboratory there has always been a nice atmosphere, and I feel very lucky that Rikke Bagger Jørgensen has been my external supervisor and Bente Anni Andersen has been the chief of the laboratory as they are competent, involved, optimistic and encouraging and I want to thank them both warmly for that. I am also thankful to my internal supervisor Marianne Philipp (University of Copenhagen) for discussion on the project during the study as well as comments on papers.

Very many people have been supportive and helpful, providing me the time and resources necessary to accomplish this study. My husband Reno has been unique and I owe a special thanks to my sister Janne, mother Hanne and friend Heidi.

Summary

In species where chloroplast inheritance is exclusively or predominantly maternal, pollen-mediated movement of transgenes is reduced or excluded if transgenes are inserted in chloroplast DNA (such plants are referred to as transplastomic) instead of nuclear DNA. However, transmission of chloroplast-encoded transgenes will still occur if transgenic individuals act as the maternal parent when hybridisation and backcrossing takes place, but the transgenes will only follow the seeds and not the pollen.

The inheritance of chloroplasts in the crop-wild model-system *B. napus* and *B. rapa* is maternal both within and between the two species. Irregular inheritance of chloroplast DNA has been observed in the genera *Festuca* and *Lolium*, not when F_1- hybrids between the species were produced, but when the F_1-hybrids were parents in subsequent crosses. With species-specific chloroplast markers it was therefore investigated how chloroplast DNA was inherited between F_1-hybrids (*B. napus* (♀) x *B. rapa*) and *B. rapa*; the second step in the introgression process of transgenes from transplastomic *B. napus* to *B. rapa*. The inheritance was maternal in all 122 examined cases.

Field trials with *B. napus* and *B. rapa* coexisting in different proportions and densities, were made to elucidate how these environmental factors affect the F_1-hybrid production on *B. napus*; the first step in the introgression of transgenes if *B. napus* is transplastomic. The paternity of 3,000 progenies produced on *B. napus* was determined as well as the fitness of *B. napus* mother plants. Higher plant density reduced the fitness of mother plants and the abundance of F_1-hybrids (at the 1:1 proportion) significantly. As to the proportion between the species, *B. rapa* was a stronger competitor than *B. napus*, which affected both the vegetative and reproductive fitness in *B. napus*, and increased the hybridisation frequency. The proportion seemed to be a more powerful environmental factor than the density. In conclusion, hybridisation on *B. napus* seems to be most likely at current field densities of *B. napus* and when *B. rapa* is an abundant weed.

Pod shattering from *B. napus* leads to seed-spillage, which may include F_1-hybrid seeds, have *B. rapa* stands been within or in close proximity to the cultivated area. In the field, some F_1-hybrid seeds are likely to germinate the next time *B. napus* is cultivated, and *B. rapa* seeds/plants may also still be present. Therefore, field trials with F_1-hybrids coexisting with *B. napus* and *B. rapa* were established with different proportions and densities. The paternity (F_1, *B. napus* and *B. rapa*) was determined in 1,350 progenies produced on the F_1-hybrids at the proportion with the highest abundance of F_1-hybrids and revealed that *B. napus* was the predominant father and the sirering success of the three possible fathers depended on the density. Additionally 3,000 progenies from F_1-hybrid mother plants grown at the other two proportions were screened merely for individuals sired by *B. rapa* (BC_{1r}s); the second step in the introgression process. There was a significant density effect on the production of BC_{1r}s but the effect differed among proportions and both the highest and lowest frequencies of BC_{1r}s were obtained at high plant density. Neither the proportion nor density affected the number of BC_{1r}s per square-meter significantly, however with low abundance of *B. rapa* the numbers were low and with high abundance of F_1-hybrids it was comparatively high.

The fitness of mother plants (F_1-hybrids) was determined. Biomass components decreased significantly from low to intermediate density, whereas a further increase in density only affected the thousand-kernel weight significantly. It was concluded that further introgression of transgenes from transplastomic oilseed rape to *B. rapa* (i.e. transmission from F_1-hybrids to BC_{1r}s) is most likely at current field densities of *B. napus* and when *B. rapa* is an abundant weed.

Resumé

Hos arter, hvor nedarving af kloroplaster udelukkende eller hovedsaglig er maternel, finder pollen-medieret spredning af transgener ikke sted eller reduceres betydeligt, hvis transgener indsættes i kloroplast DNA'et (planterne betegnes som transplastomiske) i stedet for kerne DNA'et. Overførsel af kloroplastkodede transgener vil dog stadig forekomme, når transgene individer fungerer som mor ved hybridisering og tilbagekrydsning. Transgenerne vil dog kun følge frøene og ikke pollenet.

Nedarving af kloroplaster i modelsystemet *B. napus* (afgrøde) og *B. rapa* (vildvoksende ukrudtsslægtning) er maternel både indenfor og mellem de to arter. Uregelmæssig nedarving af kloroplaster er observeret hos slægterne *Festuca* og *Lolium*, ikke når F_1-hybrider mellem arterne blev dannet, men når F_1-hybriderne efterfølgende var forældre i krydsninger. Ved hjælp af artsspecifikke kloroplastmarkører er det derfor undersøgt, hvorledes kloroplaster nedarves mellem F_1-hybrider (*B. napus* (♀) x *B. rapa*) og *B. rapa*; det andet trin ved introgression af transgener fra transplastomisk *B. napus* til *B. rapa*. Nedarvingen var maternel hos alle 122 undersøgte individer.

Markforsøg hvor *B. napus* og *B. rapa* sameksisterede i forskellige proportioner og densiteter, blev udført for at klarlægge om disse miljømæssige faktorer påvirker F_1-hybrid dannelsen på *B. napus*; det første trin i introgressionen af transgener hvis *B. napus* er transplastomisk. Paterniteten blev bestemt hos 3.000 afkom dannet på *B. napus*, og derudover blev *B. napus* moderplanternes fitness bestemt. Moderplanternes fitness og forekomsten af F_1-hybrider (ved 1:1 proportionen) blev signifikant lavere, når plantedensiteten blev højere. Forskellige proportioner mellem arterne viste at *B. rapa* var en stærkere konkurrent end *B. napus*, hvilket både påvirkede den vegetative og reproduktive fitness hos *B. napus* og øgede hybridiseringsfrekvensen. Proportionen var tilsyneladende en stærkere miljømæssig faktor end densiteten. Det blev konkluderet, at hybridisering på *B. napus* tilsyneladende er mest sandsynlig ved de nuværende markdensiteter, og når *B. rapa* er et hyppigt forekommende ukrudt.

Frøspild fra *B. napus* kan indbefatte F_1-hybridfrø, såfremt *B. rapa* bestande har været indenfor eller tæt på det dyrkede areal. I marken vil nogle F_1-hybridfrø sandsynligvis spire næste gang der dyrkes *B. napus*, og *B. rapa* frø/planter findes måske også stadig. Derfor blev der etableret markforsøg, hvor F_1-hybrider sameksisterede med *B. napus* og *B. rapa* i forskellige proportioner og densiteter. Paterniteten (F_1, *B. napus* og *B. rapa*) blev bestemt hos 1.350 afkom dannet på F_1-hybrider ved proportionen med den højeste forekomst af F_1-hybrider, hvilket viste at *B. napus* hovedsagelig var far, og at bestøvningssuccesen for de tre mulige fædre afhang af densiteten. Yderligere 3.000 afkom fra F_1-hybrid moderplanter dyrket ved de andre to proportioner blev screenet udelukkende for at identificere individer med *B. rapa* som far (BC_{1r}); det andet trin i introgressionsprocessen. Der var en signifikant densitetseffekt på dannelsen af BC_{1r}'ere, men effekten afhang af proportionen, og både den højeste og laveste frekvens af BC_{1r}'ere blev opnået ved høj plantedensitet. Hverken proportionen eller densiteten påvirkede antallet af BC_{1r}'ere per kvadratmeter signifikant, men når forekomsten af *B. rapa* var lav var antallet lavt og når forekomsten af F_1-hybrider var høj var antallet forholdsvis højt. Moderplanternes (F_1-hybridernes) fitness blev bestemt. Biomassekomponenterne aftog signifikant fra lav til mellem densitet, hvorimod yderligere stigning i densiteten kun påvirkede tusindkornsvægten signifikant. Det blev konkluderet, at introgression af transgener fra transplastomisk raps til *B. rapa* (d.v.s. overførsel fra F_1-hybrider til BC_{1r}'ere) er mest sandsynlig ved nuværende markdensiteter, og når *B. rapa* er et hyppigt forekommende ukrudt.

Introduction

The purpose of this introduction is to set the scene for the subsequent manuscripts. I will describe different dispersal mechanisms of transgenes with oilseed rape (*Brassica napus*) as the model crop, and *B. rapa* as the potential recipient. Different means, especially of biotechnological origin, which could reduce transgene dispersal, will be presented, with emphasis on the possibilities and difficulties when integrating novel DNA into the DNA of plastids. However, even with this approach, transgenes may escape from oilseed rape to wild relatives as *B. rapa*. By which means will be explain in detail. Then I will describe the objectives of the work presented in Paper I-V.

During my external stay for three months in the Biotechnology group, Danish Institute of Agricultural Sciences situated at the Royal Veterinary and Agricultural University, I worked with different aspects of gene-silencing, namely virus induced suppression of gene-silencing and the adjustment and implementation of a technique, which could identify ”small interfering RNAs” seen associated with post transcriptional gene-silencing. Paper VI addresses the second of these two subjects, I will however only shortly summarize the background, as it is a large research area and somewhat distant from the main subject of this thesis.

Background

Transgenic crops have been cultivated since 1995 and world wide the cultivation is increasing in several manners; more crops become available, transgene traits are slowly, however still, becoming more advanced, the number of countries approving genetically modified (GM) crops is increasing and the area cultivated with GM crops is expanding (James 2003). Studies and experience have shown that crop-to-crop gene flow can occur (Beckie *et al.* 2003; Hall *et al.* 2000). Crop to weed transgene flow has not been reported as an agricultural problem (Belzile 2002; Hall *et al.* 2000), however, the timescale may be longer for this to establish to an extent making it a problem. Several studies have nevertheless shown that coexistence between cross compatible crops and weeds may produce hybrids and backcross offspring (Jørgensen & Andersen 1994; Mikkelsen *et al.* 1996). On the world scale, transgenic crops have probably come to stay, and with that in mind as well as the increase in number of incidences of unintended transgene flow (Warwick & Meziani 2002) approaches which minimize the environmental side effects, must be considered.

Oilseed rape (*Brassica napus*) and weedy *Brassica rapa*

Oilseed rape (*Brassica napus* L.) is an amphidiploid with genome constitution AACC ($2n = 38$), believed to have arisen by hybridisation between *B. rapa* (AA, $2n = 20$) and *B. oleracea* (CC, $2n = 18$) followed by genome doubling (U 1935). Inheritance of RFLP loci have demonstrated that the nuclear genomes of *B. napus*, *B. rapa* and *B. oleracea* have remained almost unaltered since the formation of *B. napus* (Parkin *et al.* 1995; Parkin & Lydiate 1997). Oilseed rape is a partially allogamous species (Poulsen & Højland 1994) with several wild relatives (*B. rapa* (Jørgensen & Andersen 1994), *Raphanus raphanistrum* (Chevre *et al.* 1998), *B. juncea* (Frello *et al.* 1995) and *Sinapis avensis* (Warwick *et al.* 2003)). The most common transgenic trait of commercialized GM oilseed rape crops is herbicide tolerance (James 2003). Besides, GM oilseed rape with a male sterility/fertility restoration system or a high content of laurate and myristic acid in the seeds are commercialized (Nap *et al.* 2003). Due to the high oil and protein

content of oilseed rape, it holds a potential for generating a wealth of enriched or altered products.

B. *rapa* is a common weed in Denmark, occurring in oilseed rape fields, along roadsides and in other disturbed habitats (Jørgensen & Andersen 1994). It has a self-incompatibility system (Hiscock & McInnis 2003) consisting of more than hundred alleles throughout the world (Nou *et al.* 1993), which promotes outbreeding and maintains the genetic diversity.

B. *napus* and *B*. *rapa* hybridises spontaneously (Jørgensen & Andersen 1994), with a better germination of F_1-hybrids when *B*. *napus* has been taken for female (U 1935). F_1-hybrids have a triploid genome constitution AAC (2*n*=29), which include the full genome complement of *B*. *rapa*. From inheritance of RFLP loci it was demonstrated that *B*. *rapa* chromosomes were each pairing exclusively with homologous in *B*. *napus* (Parkin *et al.* 1995). The A and C genomes have homoeologous chromosome regions allowing intergenomic recombination (Chen *et al.* 1992), and (Parkin *et al.* 2003) found that almost every genetically mapped locus detected by RFLP in the A genome had a homologous locus in the C genome.

Backcross offspring with *B*. *rapa* as the recurrent parent also occur spontaneously under field conditions (Hansen *et al.* 2001; Mikkelsen *et al.* 1996). Repeated backcrossing results in gradual loss of the C chromosomes (Chen *et al.* 1990) except regions that have recombined into the A-genome. The genomes are therefore quite plastic and rather *B*. *rapa* like plants (2*n* = 20-21 and high pollen fertility) may be present among BC_1s (Mikkelsen *et al.* 1996), which gives evidence of rapid transgene dispersion.

Dispersal mechanisms

Seeds

The genes from oilseed rape can be dispersed by seed corn, volunteers from seeds lost before or during harvest (on average 5-10% of the production, but in some years up to 50% (Tolstrup *et al.* 2003) and during transportation or during handling in the production system. For example in Alberta fields, volunteer *B*. *napus* is among the 20 most common weeds and it can persist up to 4 years after sowing (reviewed by Beckie *et al.* (2003). In Denmark seeds contribute to the seed bank up to 10-12 years (Tolstrup *et al.* 2003). Volunteers and feral populations of oilseed rape may be reservoirs for genetic pollution between genetically modified and non-genetically modified crops as well as wild relatives. However, the readily established feral populations are rarely self-maintained in Denmark (Tolstrup *et al.* 2003), though in France feral plants, persisting at least 8-9 years outside cultivated fields have been recognized (Pessel *et al.* 2001). With regard to experiences with control of volunteer transgenic oilseed rape, results from a questionnaire answered by farmers in Alberta, Canada were that they mostly posed equal to or no more of a problem than management with conventional cultivars (http://www.canola-council.org/production/gmo_toc.html). Crop rotation is essential in control of volunteer plants in oilseed rape fields, as well as the soil preparation because if the soil is disturbed just after harvest, shed seeds are incorporated into the soil, where they become dormant (for more details see Tolstrup *et al.* (2003)).

Seed sterility, also designated the terminator technology, is a strategy, which prevents seeds from germinating, when some outside stimulus triggers them off. From a technological viewpoint this approach is advanced and intelligent, but it has a lot of drawbacks such as the use of an antibiotic as stimulator of seed sterility, and the

dependency of farmers on the seed producer (Steinbrecher & Mooney 1998). As to transgene dispersal, the seeds are sterile, so the problem with seed dispersal is reduced, but the pollen with seed sterilizing traits will spread.

Pollen

The genes can also be dispersed via pollen, which can be transported by wind and insects over large distances (Thompson *et al.* 1999), with insects as the primary pollen vectors (Hayter & Cresswell 2003). This makes transgenic oilseed rape an extraordinary challenge. However, different means may reduce or prevent pollen-mediated gene flow from crop fields.

Physical containment

Pollen proof environments (e.g. plastic tunnels, green houses) would prevent outflow of transgenic oilseed rape pollen as well as inflow of pollen from wild relatives as *B. rapa*. Unless a high value variety is cultivated, this will however not be economically rewarding. With respect to weed-to-crop gene flow, efficient weed control in the field and adjacent areas will provide suitable isolation distances and crop-to-crop gene flow will be reduced with increased separation distance between crops (for recommended distances see Tolstrup *et al*. (2003)). Finally, separate harvesting of border areas/buffer zones functioning as pollen traps will reduce the inflow of pollen. Based on four field trials with border areas 15-30 m wide outcrossing rates averaged 0.7% at 0 m and declined exponentially to 0.02% at 30 m, with more than four-fifths detected within the first 10 m of the border (Staniland *et al.* 2000).

Biological containment

There are different strategies to achieve biological containment of transgenes.

Male sterility prevents production of fertile pollen. It can be obtained by cytoplasmic (Bewley *et al.* 2000), nuclear (or genic, e.g. (Lu *et al.* 2004)) and transgenic (e.g. (Mariani *et al.* 1990)) means. The strategy is only applicable if the plant product of interest is not the seed, or else the male sterile type must be cultivated in mixture with a pollinating variety. Depending on the purpose (e.g. hybrid variety production) the pollinating variety may restore fertility in the seeds. The transgene should be closely linked to the gene causing sterility, ensuring co-segregation.

A second strategy, apomixis or asexual reproduction, gives seed formation without fertilization. It occurs naturally in over 400 species, however not in any major crops and the strategy is currently not feasible (Spillane *et al.* 2004). In combination with male sterility, it would provide partial transgene containment as pollen dispersal is prevented, and further a pollinating variety as with male sterility would not be needed.

Transformation of organelle DNA is a third strategy. In theory, it is possible to transform both mitochondria and plastids, but there are no reports on transformed mitochondria. One type of plastids, the chloroplast is a vital organelle responsible for photosynthesis. The concept of integration of novel genes into plastid genomes was developed in the 1980s (Daniell *et al.* 2002). This type of transgene integration has been achieved and these plants are referred to as “transplastomic” as suggested by Svab et al. (1990). Various technical approaches are available and these have been reviewed recently (Bock 2001). The efficiency of transformation is still relatively low perhaps due to the inherent

difficulty in delivering foreign DNA through the two membranes present in plastids such as chloroplasts (Belzile 2002). The main obstacle to extend the technology to several crop species is posed by limitations in the currently available tissue culture systems and regeneration protocols for transplastomic plants (Bock 2001).

Table 1 show genes, which have recently been successfully integrated in plastid DNA. They are grouped into categories including eight different marker and reporter genes, twelve plant-fitness enhancing genes and eight genes with no relation to plants.

Table 1 (Daniell et al. *2002)*

Genes and use	**Gene products and use**
Selectable markers and reporters	
AadA	Aminoglycoside-3'-adenylyltransferase
NptII	Neomycin phosphotransferase
CodA	Cytosine deaminase
BADH	Betaine aldehyde dehydrogenase
UidA	β-glucoronidase
Cat	Chloramphenicol acetyl transferase
Gfp	Green fluorescent protein
AadA:gfp	Selectable or screenable fusion protein
Plant traits: herbicide resistance	
AroA	Glyphosate resistance
Bar	Bialaphos resistance
Insect resistance	
Cry1Ac	Bacillus thuringensis (Bt) toxin
Cry2Aa2	Bacillus thuringensis (Bt) toxin
Cry2Aa2 operon	Bacillus thuringensis (Bt) toxin
Pathogen resistance	
msi-99	Bacterial, fungal resistance
Drought or salt tolerance	
Tps1	Trehalose phosphate synthase
BADH	Betaine aldehyde dehydrogenase
Amino acid biosynthesis	
EPSPS	5-enol-pyruvyl shikimate 3-phosphate synthase
ASA2	Anthranilate synthase (AS) α-subunit
Phytoremediation	
mer A	Mercuric ion reductase
mer B	Organomercurial lyase

Non-plant traits: biopharmaceuticals	
HST	Human somatotropin
HAS	Human serum albumin
msi-99	Anticancer, lytic antibiotic
Proinsulin	Human insulin α, β chains
IFN α5	Human interferon α5
Monoclonals	
Guy's 13	For dental carries against *streptococcus mutans*
Biomedical polymer	
Gvgvp-120	Bioelastic protein-based polymer
Edible vaccines	
CtxB	Cholera toxin β-subunit

The number of species with successful plastid transformation is increasing and includes tobacco (Svab *et al.* 1990), *Arabidopsis thaliana* (all regenerated plants were sterile (Sikdar *et al.* 1998)), potato (Sidorov *et al.* 1999), tomato (Ruf *et al.* 2001), *Lesquerella fendleri* (Skarjinskaia *et al.* 2003), rice (heteroplasmic (Khan & Maliga 1999)) and oilseed rape (heteroplasmic (Hou *et al.* 2003)).

A single plant cell may contain up to 100 plastids, each harbouring up to 100 genome copies (Bendich 1987) organized in nucleoids and several such nucleoids are present in each chloroplast (Bock 2001). This gives estimates up to 10.000 genome copies per plant cell e.g. in pea leaf cell (Bendich 1987) and even up to 50,000 copies in wheat (Bendich 1987), though, the usual range is about 5.000-10.000 per plant cell (Daniell *et al.* 2002).

Each plastid genome, which is a comparatively small and circular chromosome consisting of double-stranded DNA, is in the range of 70-250 kb and contains approximately 100 genes (Belzile 2002; Herrmann 1999). Due to the many genome copies chloroplast DNA typically makes up as much as 10-20% of the total cellular DNA content (Bendich 1987).

So compared with a single gene copy per cell obtained by nuclear transformation, transplastomic crops have an extremely high transgene copy number. This allows an extremely high level of expression of the protein (De Cosa *et al.* 2001), which is 10-100 times higher than upon nuclear transgene expression in plants (Bock 2001). But it also confers an extra challenge, as an increased number of cell divisions under high selective pressure are needed to attain homoplasmy (two-to-four cycles of regeneration and selection for tobacco (Bock 2001)). Homoplasmy is a condition where all (or nearly all) plastid genomes in the cell contain the introduced gene (Belzile 2002) and it is required to avoid sorting-out due to random genome segregation upon organelle division as well as random organelle segregation upon cell division (Bock 2001). Heteroplasmic cells contain a mixture of plastid genomes with and without the introduced gene.

The insertion site is unknown when novel DNA is integrated into nuclear DNA. Conversely, homologous recombination (preceding integration, the genetic construct is flanked by > 400 bp DNA regions homologous to plastid DNA) can be exploited when novel DNA is integrated into plastid genomes, which makes the integration site precisely

known (Belzile 2002). Independent transformants are therefore identical, giving uniform expression among transgenic lines and elimination of position effects (Daniell *et al.* 2002). Unfortunately the information on plastid genome sequences for several important crop species are lacking (but see http://www.ncbi.nlm.nih.gov), however, conservation of plastid genome sequences across many plant species allow using targeting sequences from one species when genetically engineering another species (Daniell *et al.* 2002).

Gene silencing, which is the result of epigenetic gene-inactivation mechanisms, is a common phenomenon in nuclear transformants when more than one transgene copy is inserted, or when there are large (~80%, Sijen & Kooter (2000)) or complete sequence homologies between a native gene and the inserted gene. Gene silencing has not been observed in transplastomic crops (Bock 2001; Daniell *et al.* 2002).

The plastid compartment has some advantages over the cytosol. Firstly, a selection technique other than antibiotic resistance has been developed (Daniell *et al.* 2001) or a recent advance, which eliminates resistance genes subsequent to transformation, can be exploited (Daniell *et al.* 2002). Secondly proteins that are toxic in the cytosol can be expressed and accumulated in the plastids at high levels (De Cosa *et al.* 2001). Thirdly it can form disulfide bonds and fold eukaryotic proteins (Daniell *et al.* 2002).

The introduced transgenes are expressed constitutively in all plastid containing plant cells (living cells). Plastid transgene expression may however be restricted to a certain tissue or developmental stage by a combination of nuclear and plastid encoded transgenes (Bock 2001).

Other benefits of transplastomic crops are apparent. If several transgenes (e.g. complex traits, whole metabolic pathways) need to be introduced and co-ordinately regulated, then, because of the prokaryotic origin of plastids, they possess the feature that a single promoter simultaneously can regulate the expression of numerous coding regions. The resulting RNAs are polycistronic (i.e. they encode multiple proteins that are separately translated from the same mRNA molecule). Thus a complete operon may be introduced in a single transformation event (Belzile 2002).

All of the described features are technical advantages and do not directly benefit the environment (except perhaps for the high expression level of some proteins). However, the feature that most angiosperms seldom transmit their plastids via pollen (Mogensen 1996) making the plastids only or mainly maternally inherited, is an environmental benefit as the pollen-mediated gene flow to neighbouring fields or wild relatives of the cultivated species will be greatly reduced or entirely excluded. This is due either to exclusion of plastids by unequal organelle distribution in the microspore before pollen grain mitosis (Clement & Pacini 2001), by degeneration of plastids during pollen maturation or pollen-tube growth (Pacini *et al.* 1992) or at the time of fertilization, by separation of plastids from the sperm nucleus just before penetration (Hagemann & Schröder 1989), but the last-mentioned remains unclear in most species (Clement & Pacini 2001). In *B. napus* all plastids of the microspore are passed on to the vegetative cell of the pollen grain (Murgia *et al.* 1991). As to the high prevalence of maternal inheritance among angiosperms Mogensen (1996) suggests that it perhaps evolved as a mechanism preventing foreign or pathogenic DNA from entering the egg, and male organelles just happened to fall into that category; the endosymbionts that evolved to plastids once were foreign. However, the development of a specific type of plastid inheritance was probably affected by several different factors. This is reviewed in more detail by Mogensen (1996).

There has been a case with oilseed rape plants acquiring three resistance genes against broad-spectrum herbicides, through pollen exchange between three cultivars with nuclear encoded herbicide resistance grown in close proximity (Hall *et al.* 2000). Because of maternal inheritance, transgenes integrated in plastids will not or seldom contribute to such gene-stacking.

Transgene dispersal from transplastomic oilseed rape

The above-mentioned strategies for biological containment are not exhaustive as to the possibilities for minimizing gene flow, but they are very often met in the literature. In my research project, I chose to focus on transplastomic oilseed rape, even though the strategy was in its childhood. I thought it was a rational alternative to nuclear transformation; even if the containment was not complete (uniparental maternal inheritance) the pollen-mediated transgene dispersal would be considerably reduced. Why not exploit that and spare the environment?

However, with this strategy, there are still possibilities for transgene dispersal; seed dispersal is not avoided as well as the inflow of pollen from adjacent crops or wild relatives. The possible route for introgression of transgenes integrated in plastid DNA of oilseed rape into *B*. *rapa* is illustrated in Figure 1. With *B*. *rapa* as the recurrent paternal parent the transgene will eventually be transmitted from *B*. *napus* to *B*. *rapa*, and transplastomic *B*. *rapa* will arise.

Hybridisation with *B*. *rapa* as the paternal parent is also present when crops with genetically modified nuclear DNA are cultivated, but in general, hybridisation with oilseed rape as the maternal parent has not attained as much focus as hybridisation with *B*. *rapa* as the maternal parent. Therefore, there are few available data about the transgene dispersal relevant with respect to cultivation of transplastomic oilseed rape. In addition, most experiments on interspecific gene flow have been made with spring types of oilseed rape; they have a short growth period and need no vernalization. Nevertheless, winter types of oilseed rape are more relevant at least from a Danish/European perspective as they cover a larger proportion of the agricultural area. Winter types are more robust and branched, with a resulting higher yield. Thus if the frequency of hybridisation is the same on spring and winter types more F_1-hybrids will be produced on winter types. Hybridisation on winter types is also likely to produce F_1-hybrids, which have higher vegetative and reproductive fitness (larger pollen production and seed set per plant) than F_1-hybrids produced on spring types. This may increase the likelihood of the subsequent steps in the introgression process. Fitness of the mother plant (oilseed rape), the F_1-hybrids and further backcross generations is an important factor for the introgression of transgenes. The environment is decisive for the fitness, and among environmental factors, the number and type of competitors are crucial.

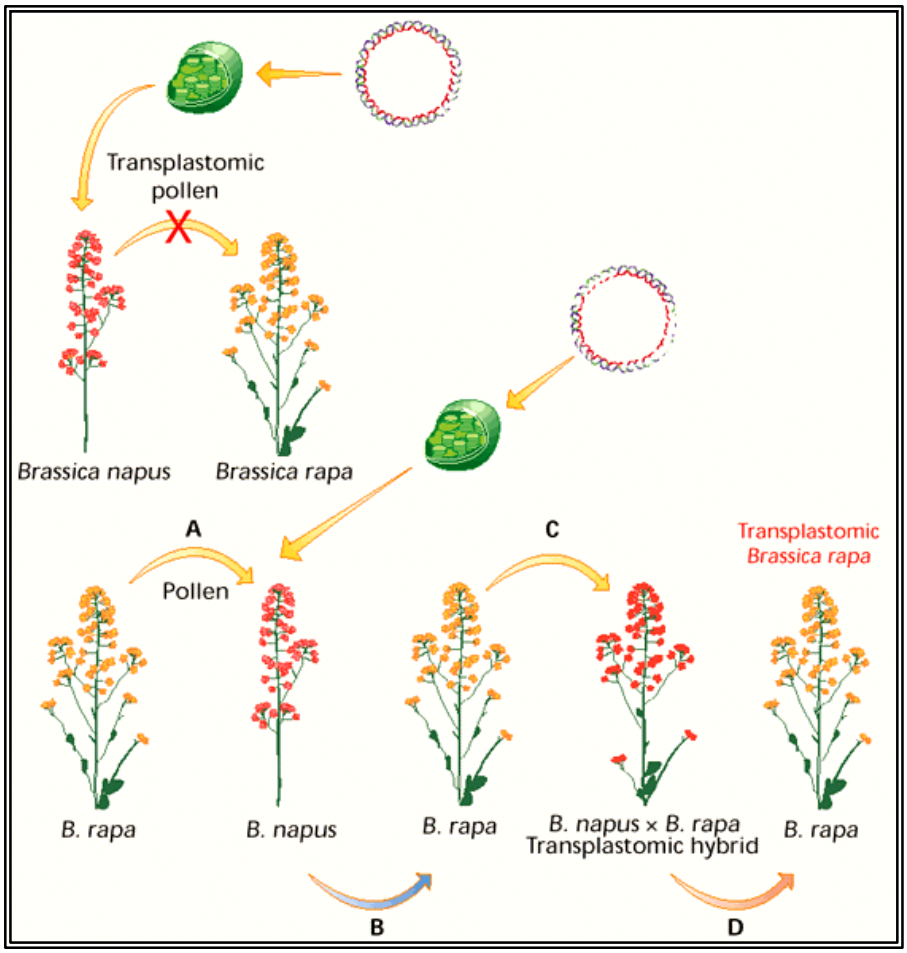

Figure 1 Illustration of the possible transgene introgression from transplastomic oilseed rape to B. rapa *(Chamberlain & Stewart 1999)*

Objectives of the study

The experimental work of the Ph.D. thesis was designed to enlighten the issues described in the following.

Since the inheritance of chloroplasts between *B. napus* and *B. rapa* is uniparental maternal (Scott & Wilkinson 1999), hybridization with *B. napus* as the seed parent plant is the only transgene escape route into *B. rapa* when cultivating transplastomic varieties. Knowledge about the extent of hybridization on *B. napus*, the first step in the introgression process, is necessary for the evaluation of risks associated with cultivation of transplastomic oilseed rape. A field trial was designed with three different densities overlapping those applied during commercial cultivation and two different proportions between a winter variety of oilseed rape and a weedy *B. rapa* population. The effect of the environmental factors on the frequency of F_1-hybrids per mother plant, the number of F_1-hybrids per square-meter (takes the seed set per mother plant into consideration) and the fitness of mother plants was determined. Results are presented in Paper I. Paper V (book chapter) was published contemporary with the conclusive meeting in the Danish

Centre for Bioethics and Risk Assessment (CeBRA). It presents results from the same work as just described, however at a time where the results were preliminary, which explains why there are not absolute consistency between the results presented in the papers. Paper V has only been published in Danish and is written in an informal style since the purpose of the paper was to inform lay people about the results obtained in CeBRA.

The inheritance of chloroplasts, is known to be strictly maternal within (Corriveau & Coleman 1988; Erickson & Kemble 1990) and between (Scott *et al.* 1999) *B. napus* and *B. rapa*, however the second step in the introgression process – formation of backcross plants - involves F_1-hybrids and here the regularity of chloroplast inheritance is unknown. F_1-hybrids could be a hot spot for biparental or paternal inheritance of chloroplasts as an abnormal nuclear background is obtained in this generation. Besides, irregular inheritance has previously been observed in crosses when intergeneric hybrids were backcrossed as follows: (*Festuca pratensis* (♀) x *Lolium perenne* (♂)) (♀) x *Lolium perenne* (♂) (Erickson & Kemble 1990). Reciprocal controlled crosses between F_1-hybrids (*B. napus* (♀) x *B. rapa*) and *B. rapa* were performed to elucidate possible irregularities. Irregularities with F_1-hybrids as father would be important to quantify, as that would affect the containment effect obtained by cultivating transplastomic oilseed rape. Irregularities with F_1-hybrids as mother would on the other hand result in a substitution of genetically modified chloroplasts with unmodified ones. Molecular DNA markers were developed for the identification of chloroplasts and the markers were further characterized by examining their distribution in different oilseed rape varieties including some known to harbour *B. rapa* chloroplasts and in representatives of *B. oleracea*. Results are presented in Paper II.

Knowledge about the extent of backcrossing on F_1-hybrids with *B. rapa* as the male parent, the second step in the introgression process, is necessary for the evaluation of risks associated with cultivation of transplastomic oilseed rape. For the second step to occur, F_1-hybrids (*B. napus* x *B. rapa*) and *B. rapa* have to coexist at some point. That is likely to occur the next time oilseed rape is cultivated in the field, where the F_1 hybrid was formed. It was therefore natural to design a field experiment were F_1-hybrids and *B. rapa* were coexisting together with a new winter variety of oilseed rape than the maternal parent of the F_1-hybrids. Crop rotation would introduce a time gap between subsequent oilseed rape cultivation making cultivation of the same oilseed rape variety unlikely. Different winter varieties were therefore included in step one and step two of the introgression process. The environmental effects of the same three densities and three different proportions (*B. napus*:*B. rapa*: F_1-hybrids) on the frequency of BC_1s and the number of BC_1s per square-meter produced on F_1-hybrids with *B. rapa* as the recurrent paternal parent were determined, as well as the environmental effect on the fitness of mother plants (F_1-hybrids). At the proportion with the highest relative abundance of F_1-hybrids the paternity was assessed in all investigated offspring. From this it was possible to reveal if some fathers (*B. napus*, *B. rapa*, F_1-hybrids) were more frequent and if the density affected the preference of fathers. Results are presented in Paper III.

Paper IV was a refereed chapter in a book published as an outcome of symposium in Amsterdam 2003 held under the title: "Introgression from genetically modified plants into wild relatives and its consequences". The paper presents part of the work performed by the Ecological Risk Assessment Group at Risø National Laboratory, which I have been part of during the study. I developed several of the markers used during identification of hybrids e.g. the ISSR and AFLP markers, as well as I contributed by critically reading the paper, applying the skills I have acquired during the study.

Post-transcriptional gene-silencing, "small interfering RNAs"

Finally, I will shortly introduce "small interfering RNAs", which is seen associated with post-transcriptional gene-silencing. I contributed with the adjustment and implementation of the technique used for the identification of the "small interfering RNAs" reported in Paper VI. Post-transcriptional gene-silencing (PTGS) is a phenomenon which begins after the introduction of a gene (i.e. virus infection or genetic engineering) that is homologous to an endogenous gene or by co-introduction of two or more homologous genes. The single most important feature of post-transcriptional gene-silencing shared between kingdoms is the formation of small (21-23 bp) interfering RNA molecules (siRNAs) (Susi *et al.* 2004). The molecules are derived from the target gene dsRNA by a nuclease. The amount of siRNAs correlate with the extent of PTGS (Hamilton & Baulcombe 1999) and the production of siRNAs initiates a process, which can result in systemic silencing.

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Paper I

Competition affects transmission of transgenes from transplastomic oilseed rape to weedy *Brassica rapa*

(Submitted to *Heredity*)

Competition affects transmission of transgenes from transplastomic oilseed rape to weedy *Brassica rapa*

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Abstract

Under the assumption that plastids are inherited uniparental maternally, F_1-hybrid production on oilseed rape is the only transgene escape route when cultivating transplastomic oilseed rape. We investigated the F_1-hybrid production on winter oilseed rape co-cultivated with weedy *B*. *rapa* at three plant densities and with two proportions between the two species. Thus, contrary to most studies on hybridisation between oilseed rape and *B*. *rapa*, this study was focused on hybridisation with oilseed rape as the maternal parent. The paternity of the oilseed rape progeny was assessed. In order to correlate hybridisation and plant competition, several fitness parameters were determined in oilseed rape mother plants.

With higher density, the vegetative fitness per mother plant decreased significantly. The density only affected the abundance of F_1-hybrids significantly (decrease) at the 1:1 proportion. As to the proportion, more *B*. *napus* plants in the field significantly decreased the production of F_1-hybrids. More *B*. *napus* and less *B*. *rapa* also increased most biomass components significantly. Thus, *B*. *rapa* was a stronger vegetative competitor than *B*. *napus*, which affected both the vegetative and reproductive fitness in *B*. *napus*, and increased the hybridisation frequency. In conclusion, the proportion was apparently a more powerful environmental factor than the density, and it appears that hybridisation on *B*. *napus* is most likely at current field densities of *B*. *napus* and when *B*. *rapa* is an abundant weed.

Introduction

Cultivation of genetically modified crops should be manageable in a way minimizing unwanted effects on the natural and cultivated ecosystems, and the farmer's free right to choose among crops and management form should be maintained. One of the large obstacles to integrate GM crops in the cropping system is their flow of transgene-containing pollen to the surroundings. It would therefore be of great value to remove transgenes from pollen. In most angiosperms inheritance of plastids are only or mainly maternal (Mogensen, 1996), thus integrating transgenes in plastid DNA (transplastomic is the term used to distinguish transgenic plants with genetically modified plastids from those where the transgene is inserted into the nuclear genome (Svab *et al*, 1990)) would decrease transgene dispersal from these species. Consequently, adventitious mixture of transgenes in the harvest from non-GM fields, outcrossing to wild relatives and transgene stacking would be reduced.

Plastid transformation has succeeded in a growing number of higher plants including tobacco (Svab *et al*, 1990), *Arabidopsis thaliana* (Sikdar *et al*, 1998), potato (Sidorov *et al*, 1999), tomato (Ruf *et al*, 2001), *Lesquerella fendleri* (Skarjinskaia *et al*, 2003) and oilseed rape (Hou *et al*, 2003). Marker and reporter genes, genes encoding plant-fitness enhancing traits and non-plant traits have been successfully integrated (reviewed by Daniell *et al* (2002)).

Unfortunately, even with strict maternal plastid inheritance the transgene containment is not complete, as seeds from the transplastomic crop will be spilled. In addition, if a wild species sires the transplastomic crop then transplastomic F_1-hybrids will be produced, and further, if the wild species acts as the recurrent paternal parent in subsequent generations, then, eventually, the transgene will be transmitted from the crop to the wild species (Chamberlain and Stewart, 1999).

Transplastomic oilseed rape would be of great convenience. Oilseed rape is rather out-crossing and hybridises spontaneously with wild relatives as *Brassica rapa* (Jørgensen and Andersen, 1994) and *Raphanus raphanistrum* (Chevre *et al*, 1998). Both wind and insects are pollen vectors, which make pollen flow hard to control. Oilseed rape is a common weed in Danish arable fields with inefficient weed control, it appear as volunteer in fields, and as a ruderal plant, however, the readily established feral populations are rarely self-maintained in Denmark (Tolstrup *et al*, 2003). Seeds are spilled before and at harvest (on average 5-10% of the production, but in some years up to 50%), and contribute to the seed bank up to 10-12 years (Tolstrup *et al*, 2003). As regards plastid inheritance plastid DNA has not been found in the generative and sperm cells of *B. napus* (Corriveau and Coleman, 1988), which is in accordance with the finding that chloroplast DNA inheritance in *B. napus* was maternal (Erickson and Kemble, 1990).

B. rapa is a common weed in Denmark, occurring in oilseed rape fields, along roadsides and in other disturbed habitats (Landbo *et al*, 1996). *B. napus* and *B. rapa* are cross compatible and hybridisation with *B. rapa* as seed parent has received much attention (e.g. Pertl *et al*, 2002; Warwick *et al*, 2003) contrary to hybridisation with *B. napus* as seed parent (Jørgensen and Andersen, 1994; Hauser *et al*, 2003). However, since hybridisation with *B. napus* as the seed parent is the only or main transgene escape route into *B. rapa* when cultivating transplastomic varieties it is important to estimate the extent of hybridisation on this species.

We wanted to throw light on the following questions:

- Is the F_1-hybrid production on *B. napus* influenced by different proportions and densities between *B. napus* and *B. rapa*?
- Do different proportions and densities change the growth conditions and consequently the vegetative and reproductive fitness of *B. napus*?

We have estimated the F_1-hybrid production on a winter variety of oilseed rape, co-cultivated in the field with weedy *B. rapa* in two different proportions and three different densities. The F_1-hybrids were identified by their genetic fingerprint; progenies harvested on *B. napus* with *B. rapa* specific DNA-markers were F_1-hybrids. To reveal the effect of plant competition on vegetative and reproductive fitness (e.g. interspecific hybridisation) several fitness parameters were determined on *B. napus*.

Materials and methods

Field trial

Transplastomic oilseed rape varieties were not available, thus we chose the conventional oilseed rape variety Capitol (winter type, produced through the doubled-haploid technique by "Cargill", France). It was sown in August as a monoculture and grown under natural environmental conditions. Seeds of weedy *B. rapa* consisting of intercrosses between four wild populations collected at Zealand were germinated according to treatment 3 described by Landbo and Jørgensen (1997). After 3-10 days, germinated seeds were transplanted into soil filled trays and cultivated in the greenhouse until the 3-4 leaves stage. In the middle of May, both species were transplanted to the experimental fields. The flowering time of the two species was synchronized (some *B. rapa* plants were disbudded), as they have a considerable overlap in flowering time under natural circumstances. Plots were established with oilseed rape and *B. rapa* coexisting in a 3:1 (*B. napus*:*B. rapa*) proportion corresponding to a realistic scenario in Danish agricultural areas and a 1:1 proportion as a worst-case scenario. The recommended field density of oilseed rape is 60-90 plants/m^2. Lower densities increase the risk of yield loss (caused by inadequate exploitation of the agricultural area), whereas higher densities increase the risk for elongation growth in the autumn. Under good growth conditions 40 plants/m^2 is acceptable (Danish Agricultural Advisory Service, Århus, Denmark). We chose densities of 16 plants/m^2 (25 cm between plants), 44.5 plants/m^2 (15 cm between plants) and 100 plants/m^2 (10 cm between plants), giving six plots in all. Plots with the same proportion, but different densities, were separated by 1 m, whereas the two proportions were separated by 10 m of winter wheat. Plants were planted with equal distance to comply the densities and in specific patterns to ensure that the proportions were accomplished. The plots were quadratic and included 150 *B. napus* and 50 *B. rapa* plants in the 3:1 proportion and 50 plants of each species at the 1:1 proportion.

Plants were sprayed regularly against pollen beetles (*Meligethes aeneus*) through the flowering period. After eleven weeks the seeds on *B. napus* were mature and plants inside the two outermost rows surrounding the plots were harvested. The plants were cut just above the ground and left to dry in open paper-bags. Ten *B. napus* mother plants from each plot were selected and 50 seeds from each mother plant were germinated,

giving 3000 progenies for analysis of paternity. Also, the vegetative dry-biomass, the number of pods, the yield (total seed weight) and the seed number were determined.

DNA extraction, SSR and Inter-SSR analysis

DNA was extracted from leaves by the simple alkaline extraction technique described by Milligan (1998), with the following modifications: 200 µl 0.5 M NaOH were added to a 1-2 cm^2 leaf sample in a micro tube and grinded with two steel beads in a mixer mill. 20 µl extraction-suspension was transferred to 480 µl storage buffer.

The SSR-PCR was made using the procedure described by Szewc-McFadden *et al* (1996) with modifications as follows: 2 µl extracted DNA, 1 U *Taq* DNA polymerase (Promega) with 1 X Reaction Buffer A (provided with the enzyme) and 2.0 mM $MgCl_2$. PCR was made with the primer pair B.n. 12A (Szewc-McFadden *et al*, 1996). PCR amplifications were performed on a Techne Touchgene Thermocycler (Buch & Holm A/S) following the program described by Szewc-McFadden *et al* (1996).

The Inter-SSR-PCR was made according to the procedure described by Charters *et al* (1996) with modifications as follows: 5 µl extracted DNA, 1 U *Taq* DNA polymerase with 1 X Reaction Buffer A and 2.0 mM $MgCl_2$. PCR was made with the degenerate primer 888 (BDB-[CA_7]) (Charters *et al*, 1996). PCR amplifications were performed on a Techne Genius Thermocycler (Buch & Holm A/S) and the program described by Charters *et al* (1996) was applied.

After the PCR, 8 µl formamide loading buffer (bromphenol blue xylen cyanol dye solution, Sigma-Aldrich) was added to the reaction products. The samples were heated at 96°C for 5 minutes and then quickly cooled on ice. Samples were multiplexed in the loading-process consisting of 1 µl SSR and 4 µl Inter-SSR product and loaded on a 3% polyacrylamide gel. The PCR-products were electrophoresed and visualized by silver staining after the methods described by Johannessen *et al* (2002).

Data analysis

We have formulated a hypothesis as follows:

The composition (proportion) and density of plants are environmental factors changing the competitive conditions. Changes in the competitive conditions are reflected in the vegetative fitness, which has an effect on the reproductive fitness. Both vegetative and reproductive fitness affects hybridisation; therefore these environmental factors have an effect on the frequency of F_1-hybrids per mother plant and the number of F_1-hybrids per square-meter.

Data were subjected to analysis of variance (ANOVA, software: SAS version 8.2, SAS Institute Inc., Cary, NC, USA). The analyses were made with the dependent variables as follows: the frequency of F_1-hybrids per mother plant and the number of F_1-hybrids per square-meter. The analyses were also made with the pod-number, the yield, the seed-number and the dry-biomass as the dependent variables since these biomass components express the vegetative fitness of the oilseed rape mother-plants. The analyses involved the proportion and density of plants in the field as factors. With the frequency of F_1-hybrids and the number of F_1-hybrids per square-meter as dependent variables the pod-number, yield, seed-number and dry-biomass were included in the analyses as covariates. Pod-number, seed-number and dry-biomass were also included as covariates when the analyses were made with the dependent variable yield.

Results

Markers for identification of F_1-hybrids and calculation of frequencies of F_1-hybrids

The fingerprinting techniques produced three dominant markers, ISSR-1, ISSR-2 and SSR-1, specific to the *B. rapa* population used in this experiment. Since none of the developed markers were monomorphic and homozygous in 100 individuals of a *B. rapa* test sample some F_1-hybrids could remain undetected. To compare the marker frequencies in known F_1-hybrids with F_1-hybrids from field, marker analyses of 100 F_1-hybrids obtained by controlled crosses between *B. napus* (♀) x *B. rapa* (♂) was performed. The controlled crosses were made with ten *B. napus* and 100 *B. rapa* plants of same parental origin as in the field experiment. *B. napus* was emasculated and bumble-bees made the pollinations. The markers were not different alleles at the same locus as all kinds of permutations between the three markers were represented in the *B. rapa* test sample, as they were in the F_1-hybrids from controlled crosses and the F_1-hybrids from the field. The marker frequencies are given in Table 1.

The assumption that the markers should be selectively neutral was however undermined for two of the markers. The ISSR-1 marker present in 26% of the *B. rapa* test sample with an expected inheritance of 13-26% (heterozygous-homozygous in *B. rapa*) to the F_1-hybrids was apparently selected against as it was only present in 8% of the F_1-hybrids from the controlled cross. The SSR-1 marker present in 73% of the *B. rapa* test sample, was observed in 41% of the F_1-hybrids from the controlled cross, which was within the expected range of 37-73% (heterozygous-homozygous in *B. rapa*). Nevertheless, out of the 59 identified F_1-hybrids from the field 51 had the marker, giving 86%. Even if 25% of the individuals in the field had remained undetected (as in the controlled cross), then 51 out of 1.25*59 individuals, equaling 69%, was still diverging too much from 41% for a neutral marker, about which it is assumed that the proportion of homozygotes and heterozygotes is about the same in F_1-hybrids from the field and the controlled cross. The ISSR-2 marker present in 95% of the *B. rapa* test sample would be expected to be present in 48-95% (heterozygous-homozygous in *B. rapa*) of the F_1-hybrids. In the controlled cross 53% of the F_1-hybrids had the marker and in the F_1-hybrids from the field 34 out of 59 or out of 1.25*59, corresponding to 58% or 46%, should have the marker. The frequencies of this marker in the F_1-hybrids from the controlled crosses and the F_1-hybrids identified in the field corresponded well, and they were within the expected range for a neutral marker inherited from *B. rapa* to F_1-hybrids. Based on these considerations, only progenies with the ISSR-2 marker were included in the data analysis. The frequency of ISSR-2 in the F_1-hybrids from the controlled crosses was used to estimate the total number of F_1-hybrids in the field. Since 53% of the F_1-hybrids in the controlled cross, had the marker, 47% of the F_1-hybrids from the field were undetected by our techniques. Therefore, the correction factor was n/53 x 100, with n = the number of identified F_1-hybrids among the fifty progenies investigated per mother plant.

F_1-hybrids per mother plant and per area

Out of the 50 progenies investigated, each mother plant produced 0-4 F_1-hybrids at the 1:1 proportion and 0-1 F_1-hybrids at the 3:1 proportion. F_1-hybrids were identified in 50-80 % of the mother plants at the 1:1 proportion and 10-20 % of the mother plants at the 3:1 proportion.

The frequency of F_1-hybrids was significantly ($p \leq 0.05$) higher at the 1:1 proportion than the 3:1 proportion (Table 2 and Table 3). Improved explanation of the data was not

accomplished by other models. When the density was increased, the frequency of F_1-hybrids increased at the 1:1 proportion and decreased at the 3:1 proportion. The lowest frequency was obtained at intermediate and high density at the 3:1 proportion.

From an agricultural viewpoint, the F_1-hybrid production per area unit is an interesting quantity, because it takes plant density and seed number into account. The average number of F_1-hybrids per square-meter is presented in Table 2. The average number of F_1-hybrids per square-meter was significantly higher at the 1:1 proportion (Table 3). The density, the interaction between density and proportion and the covariates pod-number, dry-biomass and seed-number did not improve the explanation of the data. When only analysing one factor at a time a significant density effect on the number of F_1-hybrids per square-meter at the 1:1 proportion and a significant effect of proportion on the number of F_1-hybrids per square-meter at the intermediate density were revealed by t-tests (results not shown). The remaining t-tests were not significant. The frequency of F_1-hybrids was largest at the high density and the 1:1 proportion whereas the number of F_1-hybrids per square-meter was largest as well at the 1:1 proportion but at intermediate density.

Biomass components

Mean values of biomass components are presented in Table 4. Except for the 1000-kernel weight, which apparently is a conserved trait in oilseed rape, there was a significant decrease in the biomasses as the density was increased. The proportion between *B*. *napus* and *B*. *rapa* also affected the production of the biomass components, which was smaller at the 1:1 proportion compared with the 3:1 proportion. The difference was significant for the yield, the seed-number and the total biomass (Table 5).

Discussion

Selection of markers for F_1-hybrid evaluation

In the controlled crosses, *B*. *napus* plants were damaged when anthers were removed, but otherwise the conditions were controlled and optimal. In the field, plants were affected by numerous uncontrolled factors. These different conditions perhaps favoured different genes, thus only selectively neutral markers would have the same frequencies in the controlled and spontaneously produced F_1-hybrids.

When we used DNA markers as a diagnostic tool for identification of specific genotypes, the markers were assumed selectively neutral. This feature was not fulfilled in two out of the three markers, as the frequencies were not corresponding between F_1-hybrids from the controlled crosses and the field, or were not inherited from the *B*. *rapa* test sample to the controlled F_1-hybrids within the expected range of frequencies for a neutral marker. Based on our experience we emphasize that the validity of markers as neutral should be verified by controlled crosses before they are used as diagnostic tools.

The distribution of F_1- hybrids among mother plants

Hybridisation can be very genotype dependent (Pertl *et al*, 2002), thus to minimize the genotype noise we chose Capitol which should be rather homogenous. The homogeneity of Capitol was confirmed in our previous study (Johannessen *et al*, 2002). The *B*. *rapa* population may however have been rather diverse, as *B*. *rapa* is an outbreeding species and the population was a mixture of four different collections. Since insects are the

primary pollen vectors in oilseed rape (Hayter and Cresswell, 2003) adjacent *B. rapa* plants may have contributed the most to the pollination of each *B. napus* plant. One mother plant produced four F_1-hybrids, and the others produced fewer, thus no remarkable deviations in F_1-hybrid production was attained by the mother plants.

Effects of density

The mean value of most biomass components per mother plant (Table 4) decreased significantly as density was increased. A great effect of plant density on yield of individual plants of winter oilseed rape has previously been documented. Yield is most stable when plants are evenly distributed (Diepenbrock, 2000; Huhn, 1998). Our field experiment was established very precisely, reducing varying intra-plot-density.

During 1996-2000 the average yield under Danish growth conditions was 40.7 hkg/ha for Capitol (Pedersen, 2000), giving a yield of 4.5-6.8 g per plant with densities of 60-90 plants/m^2 as recommended. The plots that were most alike (intermediate to high density and 75% *B. napus*) performed rather well and within the same range.

The 1:1 proportion

When *B. napus* and *B. rapa* were equally represented (1:1 proportion), the frequency of F_1-hybrids increased significantly as the density increased. Thus, intensified species interaction perhaps increased the pollination advantage of *B. rapa*. In a 1:1 species mixture with a plant density of 20 plants per square-meter 9% F_1-hybrids were produced on *B. napus* (spring type) (Jørgensen and Andersen, 1994). This 3-4 times higher percentage of F_1-hybrids was found even though winter types of oilseed rape have a larger overlap in flowering time with *B. rapa*. However, Jørgensen and Andersen (1994) used more and different genotypes and the environmental conditions were different, which may have improved the conditions for outcrossing.

Since the F_1-hybrid production increased with reduced biomasses, the intensified competition, besides effects on the vegetative fitness, maybe affected the relative pollen contribution from *B. napus* and *B. rapa*. *B. napus* should have been expected to overcome a relative increase in *B. rapa* pollen since *B. rapa* pollen in *B. napus* (spring type) styles has significantly lower fitness than conspecific pollen (Hauser *et al*, 1997). In pure intraspecific pollinations, the pollen germination and growth takes 2h in *B. napus* and 4h in *B. rapa* to reach the ovaries (Röbbelen, 1960), thus as stated by Hauser *et al* (1997) it seems likely that *B. rapa* pollen in *B. napus* styles would be outcompeted by the conspecific pollen. Nevertheless, there may be a limit to the superiority of *B. napus* pollen. The significant reduction in the reproductive fitness expressed by the seed-number was therefore probably resulting from both reduced vegetative and reproductive fitness (i.e. fewer branches, fewer racemes, fewer flowers, less pollen).

The largest coefficients of variation were obtained at intermediate density. Studies made with soybean, turnip and radish showed no apparent correlation between the coefficient of variation and changes in density as reported by Kira *et al* (1953). They therefore deduced that intensified competition was quite uniformly shared among all plants and the population as a whole reacted to the limited supply of growth factors. The coefficient was affected in our experiment and the difference may be ascribed to the presence of *B. rapa* and the effect of interspecific competition.

The 3:1 proportion

There was a non-significant density-effect on the hybridisation frequency at the 3:1 proportion, despite a significant decrease in mean values of most biomass components per mother plant (Table 4). The effect of density on *B*. *rapa* and *B*. *napus* was probably equivalent, perhaps resulting in the same fitness ratio between *B*. *napus* and *B*. *rapa*.

The coefficient of variation increased with density, so the variation in biomass components increased even though the components on average became smaller. Thus, intensified competition resulted in a more diverse response among individual plants. In pure plots of soybean, turnip and radish the effect of competition are shared uniformly among individuals (Kira *et al*, 1953). During free competition the effect is shared inconsistently, thus the process of free competition perhaps acted in the present study. If free competition was part of the increased variation, then as suggested for the 1:1 proportion, it maybe relied on the presence of *B*. *rapa* and the effect of interspecific competition.

The effect of proportion

The frequency of F_1-hybrids was significantly lower at the 3:1 proportion, probably because *B*. *rapa* contributed less to the pollen being dispersed. Proportionality with the abundance of *B*. *rapa* would reduce the frequency of F_1-hybrids per mother plant from the 1:1 proportion to the 3:1 proportion with 50%. This almost agreed at low density, but at intermediate and high density, the frequency was reduced by far more than 50%. Therefore, as the density was increased, the proportion became progressively more important.

Most biomass components per mother plant were significantly or almost significantly larger at the 3:1 proportion. A spring type of *B*. *napus* likewise produced many more seeds in mixed plots with high frequency of itself (Pertl *et al*, 2002; Hauser *et al*, 2003).

The total biomass is regulated more precisely than the numerical size of a population and is thus expected to be the best measure of competition (Begon *et al*, 1990). *B*. *napus* was thus exposed to the most intense vegetative competition at the 1:1 proportion at high density when the frequency of F_1-hybrids per mother plant was also highest, and the number of F_1-hybrids per square-meter among the highest. The least intense competition was present at the 3:1 proportion at low density when the frequency of F_1-hybrids and the number of F_1-hybrids per square-meter were among the lowest. The total biomass was lower at the 1:1 proportion, *B*. *rapa* was therefore a stronger (vegetative) competitor than *B*. *napus* affecting both the reproductive and vegetative fitness of *B*. *napus* – or to put it in another way; the weed decreased the crop output. This is in accordance with the expectations as weeds are characterised by their superior competitive traits.

Besides the vegetative competition, the composition of the pollen-cloud probably affected the seed-number and thus the yield and total biomass. In pods containing both conspecific and heterospecific zygotes the survival of *B*. *napus* (♀) x *B*. *rapa* zygotes was estimated to 15% compared with *B*. *napus* zygotes (Hauser *et al*, 1997). Therefore, reproductive interactions may partly explain why the seed number, yield and total biomass were lower at the 1:1 proportion and significantly different among proportions. The average number of seeds per pod was, however, only slightly lower at the 1:1 proportion, and not significantly different. Zygote death therefore probably only explains a minor part of the differences between proportions.

The number of F_1-hybrids per square-meter was significantly lower at the 3:1 proportion, which was consistent with the significantly lower frequency of F_1-hybrids and seed-number per mother plant.

The coefficient of variation was in general lower at the 3:1 proportion at low and intermediate density, whereas it was opposite at the high density. This means that both density and proportion affect competition expressed by variation in fitness parameters. The frequency of F_1-hybrids and the number of F_1-hybrids per square-meter was apparently not correlated to the size of the coefficients of variation.

Concluding remarks

In conclusion, the density-effect was for most investigated fitness parameters highly dependent on the proportion, whereas the proportion-effect was, if not independent of densities, then, to a large extent, showing the same tendency within the densities, except for the coefficient of variation. The proportion was therefore apparently a more powerful environmental factor than the density.

The frequency of F_1-hybrids and the number of F_1-hybrids per square-meter were lowest when *B. napus* was cultivated at intermediate to high densities and the abundance of *B. rapa* in the field area was kept low. Therefore, cultivation of transplastomic oilseed rape varieties will probably produce least transplastomic F_1-hybrids under these environmental conditions. Happily, this is in accordance with present good agricultural practice.

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Table 1 The frequency of markers in a B. rapa *test sample and F_1-hybrids from controlled crosses, as well as the number of F_1-hybrids identified in the field with each marker. In parenthesis the total number of individuals analyzed or observed is given.*

Markers	Frequency of markers		Observed F_1-hybrids (n=59)
	B. rapa	F_1-hybrids (controlled crosses)	
ISSR-1	26 % (n=96)	8 % (n=96)	18
ISSR-2	95 % (n=96)	53 % (n=96)	34
SSR-1	73 % (n=97)	41 % (n=98)	51
No markers	2 % (n=95)	25 % (n=96)	?

Table 2 The frequency of F_1-hybrids per mother plant and the average number of F_1-hybrids per square-meter at each combination of density and proportion.

Proportion (*B. napus* (♀) : *B. rapa*)	Density					
	Low		Intermediate		High	
	Frequency per mother plant	Number per m^2	Frequency per mother plant	Number per m^2	Frequency per mother plant	Number per m^2
1:1	2.3%	579	3.8%	1614	5.3%	1210
3:1	0.8%	391	0.4%	252	0.4%	71

Table 3 ANOVA with the frequency of F_1-hybrids per mother plant and the number of F_1-hybrids per square-meter as the dependent variables.

	Frequency of F_1-hybrids			F_1-hybrids per square-meter		
Test of effect of	DF	F	P	DF	F	P
Proportion	1	16.5	0.0002	1	4.91	0.0308
Density	2	0.90	0.4111	2	0.42	0.6577
Proportion	1	5.35	0.0001	1	4.87	0.0315
Density	2	47.81	0.4047	2	0.42	0.6599
Proportion*Density	2	0.18	0.2287	2	0.79	0.4605

Table 4 Mean value and coefficient of variation (CV = SD/Mean) of biomass components (pod-number, yield, 1000-kernel weight, dry-biomass, seed–number, seeds per pod and total biomass) per B. napus *mother plant for each combination of density (low, intermediate and high) and proportion (1:1 and 3:1) (± 95% level of confidence).*

Proportion (*B. napus* (♀) : *B. rapa*)	Biomass component	Parameter	Density				
			Low		Intermediate		High
1:1	Pod-number	Mean	255 (±68)	>>	92 (±42)	>	40 (±14)
		CV	43%		74%		55%
	Yield (g)	Mean	18.2 (±5.5)	>>	6.4 (±3.2)	>	2.7 (±1.1)
		CV	49%		80%		66%
	1000-kernel weight (g)	Mean	4.2 (±0.3)	=	4.3 (±0.3)	=	4.6 (±0.2)
		CV	13%		12%		6%
	Dry-biomass (g)	Mean	30.9 (±7.1)	>>	9.6 (±4.0)	>	4.5 (±1.9)
		CV	37%		68%		67%
	Seed-number	Mean	4391 (±1248)	>>	1547 (±765)	>	599 (±249)
		CV	46%		80%		67%
	Seeds per pod	Mean	17.1 (±1.6)	=	16.0 (±1.6)	=	13.9 (±2.0)
		CV	15%		16%		23%
	Total biomass (g)	Mean	49.1 (±12.5)	>>	16.1 (±7.2)	>	7.2 (±3.0)
		CV	41%		72%		66%
3:1	Pod-number	Mean	301 (±49)	>>	120 (±36)	=	73 (±33)
		CV	26%		49%		73%
	Yield (g)	Mean	22.7 (±3.5)	>>	9.2 (±3.1)	=	5.4 (±2.8)

	CV	25%		54%		83%
1000-kernel weight (g)	Mean	4.4 (±0.4)	=	4.2 (±0.3)	=	4.3 (±0.3)
	CV	10%		10%		11%
Dry-biomass (g)	Mean	36.3 (±8.6)	>>	11.6 (±4.5)	=	7.7 (±3.5)
	CV	24%		62%		74%
Seed-number	Mean	5154 (±1381)	>>	2146 (±607)	>	1190 (±588)
	CV	27%		46%		80%
Seeds per pod	Mean	17.2 (±0.8)	=	18.4 (±1.7)	=	14.8 (±2.3)
	CV	7%		15%		26%
Total biomass (g)	Mean	59 (±8.7)	>>	20.8 (±7.6)	=	13.0 (±6.2)
	CV	24%		59%		77%

T-test, significance of the difference: = non-significant, > significant at a 5% level, >> significant at a 1% level.

Table 5 ANOVA with the pod-number, yield, 1000-kernel weight, dry-biomass, seed-number, seeds per pod and total biomass per mother plant as dependent variables

Dependent variable	Test of effect of	DF	F	P
Pod-number	Proportion	1	3.90	0.0532
	Density	2	56.18	<0.0001
Yield	Proportion	1	5.51	0.025
	Density	2	49.25	<0.0001
1000-kernel weight	Proportion	1	0.15	0.6961
	Density	2	0.74	0.4825
Dry biomass	Proportion	1	3.35	0.0725
	Density	2	78.74	<0.0001
Seed-number	Proportion	1	4.16	0.0461
	Density	2	53.42	<0.0001
Seeds per pod	Proportion	1	2.42	0.1257
	Density	2	6.83	0.0022
Total biomass	Proportion	1	4.25	0.0438
	Density	2	66.35	<0.0001

Paper II

Maternal inheritance of chloroplasts between *Brassica rapa* and F_1-hybrids demonstrated by cpDNA markers specific to oilseed rape and *B. rapa*

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Maternal inheritance of chloroplasts between *Brassica rapa* and F_1-hybrids demonstrated by cpDNA markers specific to oilseed rape and *B. rapa*

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Abstract

Controlled reciprocal crosses between *B. rapa* and F_1-hybrids (*B. napus* (♀) x *B. rapa*), giving 20 pair-crossings, were made to reveal possible irregularities in chloroplast inheritance during production of BC_1s. Despite the close relationship of chloroplasts in *B. rapa* and *B. napus* PCR-based molecular markers specific to *B. rapa* chloroplasts and *B. napus* chloroplasts were developed. Offspring from each cross were investigated and among these, we found no irregular chloroplast inheritance, since their plastid genotypes in all cases were identical to that of their mother. In oilseed rape, pollen-mediated transgene-dispersal poses a well-known risk. Our results enforce development of transplastomic oilseed rape as an approach to reduce transgene dispersal.

Introduction

Among the advantages of cultivating transplastomic (the term used to distinguish transgenic plants with genetically modified plastids from those where the transgene is inserted into the nuclear genome (Svab et al. 1990)) crops is the way chloroplasts are inherited. The majority of flowering plants studied to date transmit their plastids exclusively from the female parent to sexual progenies, but about one-third of the genera studied included species displaying some degree of biparental plastid inheritance (Mogensen 1996). In species where chloroplasts are inherited exclusively or predominantly maternally, transformation of chloroplasts will reduce the transmission of transgenes, since the pollen-mediated movement of transgenes thereby is excluded or reduced.

Recently transformation of chloroplasts succeeded in oilseed rape (Hou et al. 2003), which brings new actuality on a thorough investigation of the plastid inheritance in this species. RFLP analysis of mitochondrial DNA in intraspecific crosses of *B. napus* (but different lines) have shown that paternal mitochondria occasionally are being transferred to the offspring (Erickson and Kemble 1990). With their cytological investigation, Mcconchie et al. (1987) found that the two sperm cells in both *Brassica campestris* (*Brassica rapa*) and *Brassica oleracea* contained low numbers of mitochondria (4-34). In plant species where inheritance of mitochondria is not strictly maternal, plastid inheritance is also frequently not strictly maternal (Reboud and Zeyl 1994). Therefore, inheritance of plastids should be investigated in detail in *B. napus*, *B. rapa* and *B. oleracea* before development of transplastomic varieties of these species. This has been done by Erickson and Kemble (1990) who investigated the intraspecific inheritance of chloroplast DNA in *B. napus*. The females had the plastid encoded triazine resistance, which in all cases was inherited to the offspring, and restriction digests of plastid DNA from offspring revealed no bands of paternal specificity. In a large screening study, plastid DNA was not found in the generative and sperm cells of *B. napus*, *B. rapa*, and *B. oleracea* pollen (Corriveau and Coleman 1988), the prerequisite for uniparental maternal inheritance of chloroplasts. In *B. napus* this is because all plastids of the microspore are passed on to the vegetative cell of the pollen grain (Murgia et al. 1991). In the light of this, inheritance of plastids in interspecific crosses between *B. rapa* and *B. napus*, the species in focus in our study, would also be expected to be maternal in both directions. In 47 natural hybrids between cultivated *B. napus* and *B. rapa* maternal chloroplast inheritance was demonstrated by the use of primers specific to chloroplast DNA (Scott and Wilkinson 1999). The F_1-hybrids were harvested on *B. rapa* mother plants situated in close proximity to *B. napus* fields.

Organelle inheritance from intergeneric hybrids to the backcross hybrids ((*Festuca pratensis* (♀) x *Lolium perenne* (♂)) (♀) x *Lolium perenne* (♂)) was examined by RFLP and indicated that both uniparental maternal organelle inheritance and uniparental paternal organelle inheritance could occur in different backcross hybrids (i.e. one of each type) (Kiang et al. 1994). Since all investigated F_1-hybrids (*F. pratensis* x *L. perenne*) exhibited maternal organelle inheritance the irregular (i.e. paternal) inheritance event was probably occurring in the first backcross generation (Kiang et al. 1994). Paternal inheritance was observed even though other authors observed a lack of plastid DNA in generative and sperm cells in at least 100 pollen grains in at least 2 plants of *L. perenne* when they applied fluorescence microscopy as a rapid screening method (Corriveau et al. 1988). However, even if plastids are transmitted with pollen, they may be excluded in subsequent stages (Mogensen 1996). In the light of this irregularity and inconsistency among results, we wanted to investigate if chloroplast DNA inheritance was

regular/irregular when transplastomic F_1-hybrids (i.e. *B. napus* (♀) x *B. rapa*) coexist with *B. rapa*.

Backcrossing between F_1-hybrids and *L. perenne* only produced offspring with the F_1-hybrid as maternal parent. Since the irregularity in the *F. pratensis*-*L. perenne*-model apparently was related to the reproductive system of the F_1-generation female reproductive malfunction was revealed. We made two-way crosses, which succeeded in both directions. Each direction has different consequences for the occurrence of transplastomic BC_1s if the inheritance of plastids occasionally is irregular. With transplastomic F_1-hybrids as mother and *B. rapa* as father, irregularity linked to the female reproductive system would result in acceptance of paternal plastids, and the relative occurrence of transplastomic BC_1s would decrease. With *B. rapa* as mother, F_1-hybrids as father, and the irregularity thus linked to the male reproductive system, there would be a transmission of paternal plastids, and the relative occurrence of transplastomic BC_1s would increase. In the *F. pratensis*-*L. perenne*-model, plastid inheritance was either strictly maternal or strictly paternal; a biparental inheritance would reduce the effect of strict paternal or maternal inheritance.

Materials and methods

Plant material

Seeds of the winter variety of oilseed rape Capitol (produced through the doubled-haploid technique by "Cargill", France) were sown in a soil-filled tray. Seeds of weedy *B. rapa* consisting of a mix from four wild populations in southeast Zealand were germinated according to treatment 3 described in Landbo and Jørgensen (1997). Ten *B. napus*, vernalized for 13 weeks at 4°C, and 100 *B. rapa* seedlings were transplanted into soil filled pots and cultivated in a growth chamber in a phytotron, Risø Environmental Risk Assessment Facility (RERAF) at Risø National Laboratory. F_1-hybrids were then obtained from controlled crosses between *B. napus* (♀) x *B. rapa* (♂) by emasculating *B. napus* plants and allowing bumblebees from mini-hives to make the pollinations. The progenies from *B. napus* were all assumed to be F_1-hybrids as 100% F_1-hybrids and no matromorphs were obtained in corresponding crossings made by U (1935) as determined from chromosome counting. The F_1-hybrids were germinated, vernalized for 10 weeks at 4˚C and cultivated in a growth chamber in the phytotron, and a new sample of *B. rapa* seeds were germinated. The phytotron growth conditions were designed to imitate a traditional Danish/Nordic growth season from spring to late summer, with a daily simulation of sunrise and sunset in both light intensity and temperature. The average air temperature was 15˚C during the night and 18˚C during the day for nine weeks followed by three weeks with 20˚C during the night and 22˚C during the day. There was as similar diurnal pattern through the period with the day lasting for 16 hours and the night lasting for 8 hours. The light simulation was done by turning the 24 lamps on and off in a preset schedule.

When the plants were ready for crossings, F_1-hybrid and *B. rapa* chloroplast specific markers were not developed. Therefore 50 seedlings of each future parent were screened with primer pairs 'a'-'b', 'c'-'d' and 'e'-'f', which specifically amplify non-coding regions of chloroplast DNA (Taberlet et al. 1991), to reveal any deviating chloroplast genotypes among the potential parents. Extraction and PCRs were made according to the descriptions below, and PCR products were visualized on a 1.6% agarose gel stained with ethidium bromide. Primer pairs 'a'-'b' and 'c'-'d' each gave rise to one fragment, which was monomorphic within and between the F_1-hybrid and *B. rapa* plants. With

primer pair 'e'-'f' all F_1-hybrids likewise produced one fragment whereas *B*. *rapa* plants made two well amplified fragments; one of them identical in size to the one produced by F_1-hybrids and the other slightly smaller. Since the F_1-hybrids produced a marker pattern corresponding to that of *B*. *napus* varieties with *B*. *napus* chloroplasts and *B*. *rapa* with *B*. *rapa* chloroplasts (Hansen et al. 2003) it was reasonable to assume that chloroplasts in the F_1-hybrids were of *B*. *napus* specificity.

Since there were no signs of atypical chloroplast genotypes among any of the potential parents, we just selected 20 F_1-hybrids and 20 *B*. *rapa* plants, paired the plants and isolated one branch from each parental plant together in a pollen-tight bag. Flowers on F_1-hybrids were emasculated on ten pairs, letting F_1-hybrids act as mother, allowing *B*. *rapa* to sire the F_1-hybrids. Conversely, flowers were emasculated on *B*. *rapa* in the remaining ten pairs. We continued emasculating flowers until we had at least four-five well-developed pods per branch, but some parental combinations not readily produced progenies. The pollen-tight bags were shaken once every day. Pods were harvested when the seeds were mature. Seeds were germinated according to treatment 3 described in Landbo et al. (1997).

DNA extraction and development of cpDNA markers

DNA was extracted from leaves by the simple alkaline extraction technique described by Milligan (1998), with the following modifications: 200 µl 0.5M NaOH were added to a 1-2 cm^2 leaf sample in a collection microtube and grinded with two steel beads in a mixer mill. 20 µl of extraction-suspension were transferred to 480 µl storage buffer.

The 'e'-'f' primer pair from the screening of parents revealed a *B*. *rapa* specific marker, but the development of a *B*. *napus* specific chloroplast marker involved several different PCR-based approaches as follows. All amplifications were made on a Techne Genius Thermocycler (Buch and Holm A/S).

1. PCR products sufficiently large for subsequent digestion were amplified with alternative combinations of the chloroplast specific primer pairs than applied in the screening for parents, namely 'a'-'d', 'a'-'f', and 'c'-'f' (Taberlet et al. 1991) (amplification reaction and temperature profile, see below). The amplification products were digested with 3-6 of the restriction endonucleases *Taq* I, *Mse* I, *Hae* III, *Hpa* II, *Sau* 3AI or *Hinf* I, which have 4- or 5-base recognition sites. Products digested with one enzyme at a time were subsequently separated on a 1.4% agarose gel. Unfortunately, none of these PCR-RFLPs revealed a *B*. *napus* specific marker.

2. PCR products were then amplified with the chloroplast specific primer pairs *trn*C-*trn*D, *psa*A-*trn*S and *trn*M-*rbc*L (Demesure et al. 1995) in a reaction mixture consisting of 1.6 U *Taq* DNA polymerase (Promega Corporation), 1 x Reaction Buffer A (provided with the enzyme), 2.0 mM $MgCl_2$, 100 µM dNTP, 0.2 µM of each primer and 0.8 ng DNA. We used the amplification profile suggested by Demesure et al. (1995). For all primer pairs the resulting amplification products were very variable in band intensity among individuals and in some individuals, it was very faint or there was no product at all. This inconsistency would be an inappropriate starting point for making comparisons, thus we chose another strategy.

3. Primers specifically amplifying microsatellites in chloroplast DNA (Provan 2000) were then applied. Because of the usually high level of length-variation in

microsatellites, we were very confident that these primers would show us differences between the species. The PCR was made according to Provan (2000) with the following modifications: 2 mM $MgCl_2$, 100 µM dNTP and 0.8 U *Taq* DNA polymerase (Promega Corporation). Amplification was made according to Provan (2000). Separation on a 6% denaturing polyacrylamide-gel (19:1) did not reveal any differences. Therefore, we aimed at PCR-SSCP (single strand conformational polymorphism). We separated the fragments on a 6% non-denaturing polyacrylamide-gel (19:1) with 1xTBE. The electrophoresis was performed in a 5°C cold room for 16h at 150V. The amplified fragments were still monomorphic so we repeated the PCR-SSCP with 10% glycerol in the gel matrix to increase the DNA mobility (Chen et al. 2002), but this neither revealed a *B. napus* specific marker.

4. Finally, PCR-RFLP with the primers 'a' and 'd' developed by Taberlet et al. (1991), digestion with a*Taq* I and separation of fragments by denaturing PAGE, revealed a *B. napus* specific marker. By this procedure, a *B. rapa* specific marker was also produced allowing us to assess the plastid genotypes in the progeny by this procedure only.

The eventual PCR was made using the procedure described by (Charters et al. 1996) with some modifications, as were all PCRs made with primers developed by Taberlet et al. (1991). We used 5 µl extracted DNA, 1U *Taq* DNA polymerase (Promega Corporation) with 1 X Reaction Buffer A (provided with the enzyme) and 2.0 mM $MgCl_2$. PCR was made with the chloroplast specific primers 'a' and 'd' and the amplification profile developed by Taberlet et al. (1991). Subsequently 2.5 µl PCR product was digested in a 10 µl reaction volume consisting of 2U a*Taq* I (New England Biolabs), 1 X NEBuffer (provided with the restriction enzyme) and 0.1 mg/ml BSA. The digestion was made at 65˚C for three hours. Afterwards 4 µl formamide loading buffer (bromphenol blue xylen cyanol dye solution, Sigma-Aldrich) was added to the restriction products, the samples were heated at 96°C for 5 minutes and then quickly cooled on ice. The PCR-products were electrophoresed on a 6% denaturing gel in 150 min. and visualized by silver staining after the methods described by Johannessen et al. (2002). The *B. rapa* chloroplast specific marker was approximately 440 bp and the *B. napus* (Capitol) specific marker was approximately 455 bp.

The specificity of the markers was further characterized on five individuals from each of additional nine oilseed rape varieties, of which two with certainty contain chloroplasts derived from *B. rapa* (*cv*. Isuzu Natane and *cv*. Petranova). It has not been possible to obtain information about the chloroplast origin in the remaining varieties. Three representatives of *B. oleracea* (*B. oleracea* var. *gemmifera* DC.; *B. oleracea* var. *capitata* f. *alba* DC.; *B. oleracea* var. *botrytis* (L.)) were also included in the analysis.

Results

In Table 1, the total seed number produced by each pair of parents is presented. We decided when there were sufficient seeds to analyse. The numbers are therefore not reflecting the potential seed production per branch, and should not to be compared with each other. However, the Table shows that some of the pairs could not reproduce or had large difficulties. The number of progenies investigated resulted from the number of seeds produced, and their ability to germinate, which varied between 20-100%.

All investigated progenies with F_1-hybrids as their mother and *B. rapa* as their father only produced the marker specific for F_1-hybrids (the Capitol marker) and the progenies with *B. rapa* as their mother and F_1-hybrids as their father only produced the marker specific for *B. rapa*. Therefore, the mode of inheritance was maternal in all cases.

The 455 bp allele (Capitol specific) was consistently monomorph within varieties when present, while the 440 bp allele (*B. rapa* specific) was polymorph in some varieties, with the alternative allele being the '0' allele, and monomorph in other varieties (see Table 2). The three *B. oleracea* representatives had none of the markers. The 455 bp and the 440 bp alleles were never present simultaneously.

Discussion

One of the advantages obtained when cultivating transplastomic oilseed rape is the exclusion of or large reduction in pollen-mediated gene-flow, depending on the stringency of maternal plastid inheritance. Oilseed rape has previously been shown to inherit plastids maternally in intraspecific and interspecific (with *B. rapa*) crossings, but how this inheritance was accomplished in F_1-hybrids or further backcross generations have not been investigated. Interspecific hybrids represent a genome from each parental species. F_1-hybrids are thus the most genetically different progeny compared with subsequent backcross generations, which for every backcross will become increasingly identical to the recurrent parent. Chloroplast development and function relies both on structural and regulatory factors encoded within the nucleus (Somanchi and Mayfield 1999). It therefore seems reasonable to assume that the likelihood of odd plastid DNA transfer increases with the degree of interspecific genomic difference in hybrids. As noted by Kiang et al. (1994) the abnormal nuclear background of the F_1 and early backcross hybrids between *F. pratensis* and *L. perenne* may have promoted the inheritance of paternal organelles rather than the maternal, because elimination of loci required for maintenance of maternal organelles, maybe confers a selective advantage on the paternal organelles. As the most abnormal nuclear background is obtained in the F_1 generation, we chose to investigate plastid inheritance in this generation.

We optimised the conditions for observing irregular inheritance by allowing only a specific type of pollen. Under field conditions stigmas will often receive a mixture of pollen, and depending on the species, heterospecific pollen may have a lower fitness in the styles than conspecific pollen (i.e. *B. napus* versus *B. campestris* (*B. rapa*)) (Hauser et al. 1997). In interspecific F_1-hybrids, conspecific and heterospecific pollen cannot be defined, but pollen from one of the parental species may be preferred over the other. In the present study the parents varied in their ability to produce BC_1s and some were not able to reproduce at all. The reproductive barrier was probably partly the result of reduced hybrid fertility. Usually F_1-hybrids between *B. rapa* and *B. napus* has a reduced pollen fertility under field conditions (Jørgensen et al. 1996; Jørgensen and Andersen 1994; Pertl et al. 2002), but at the same time a high female fitness (seed set per plant) (Pertl et al. 2002). However, under a range of environmental conditions seeds per pod may be considerably lower in F_1-hybrids (2.9-9.5 (Johannessen et al., Paper III)) than in *B. napus* (13.9-18.4 (Johannessen et al., Paper I)). In hybrids between *F. pratensis* and *L. perenne* there were low pollen viability and low seed set. Sporophytic self-incompatibility in *B. rapa* may also partly have caused the low BC_1 production in some pairs (Bateman 1955) as the fathers of the F_1-hybrids and the BC_1s were obtained from the same *B. rapa* population, and by this means perhaps contained matching incompatibility alleles.

Seed dormancy is a typical feature of *B. rapa* and also of its BC_1 generation and may explain why seeds produced on some pairs had a low germination frequency (Landbo et al. 1997) thus there are still unknowns as the plastid inheritance in seeds not germinating under the offered conditions was not investigated.

As noticed by Kiang et al. (1994) the nuclear genotype may have a profound influence on mtDNA and cpDNA in general, and this may also mean that different varieties of *B. napus* or different wild populations of *B. rapa* may respond differently when their genomes are interacting in hybrids. The two BC_1s produced by Kiang et al. (1994) had different mothers; the hybrids were made with two different *L. perenne* varieties. The maternal parent may have had an effect on transmission and be the cause of the two different outcomes. In addition, the environmental conditions may affect the transmission. In *Nicotiana tabacum* L. the number of plastids (about 0-1 plastids per sperm cell) appeared to be the same or slightly higher in sperm cells produced in flowers grown under cooler greenhouse conditions, thus potentially influencing the likelihood of male cytoplasmic inheritance (Yu and Russell 1994). During our growth chamber experiment, we attempted to have environmental conditions as close to natural as possible.

B. rapa and *B. oleracea* are the progenitors of *B. napus* (U 1935). Based on several lines of evidence reviewed by Pradhan et al. (1992) they suggest that *B. rapa* and *B. oleracea* developed from the same ancestor. Among the evidence, there are close similarities in their cytoplasms including the chloroplasts. Our difficulties in developing a *B. napus* chloroplast specific marker may therefore reside in the close relationship between the cpDNA in *B. napus* and *B. rapa*. Depending on the *B. napus* cultivar, its cpDNA may originate from *B. oleracea* or *B. rapa*. RFLPs of cpDNA revealed that the *B. napus* cultivar (Regent) was closer related to *B. oleracea* (*var*. alboglabra, botrytis, capitata and italica) than to the *B. napus* cultivar BO-15 and to *B. campestris* (*B. rapa*) represented by several different subspecies, whereas the *B. napus* cultivar BO-15 had the closest relationship to the *B. campestris* subspecies (Pradhan et al. 1992). Phenograms constructed by the UPGMA method based on estimates of similarity coefficients (F) illustrated the relationships.

Because none of the markers were present in *B. oleracea* they are unlikely to be *B. oleracea* chloroplast specific. We therefore suggest that the 455 bp and the 440 bp alleles both are *B. rapa* chloroplast specific, however with specificity for different types of *B. rapa* chloroplasts due to the mutual exclusion of the alleles. The frequent polymorphic nature of the 440 bp allele reveals that these varieties may contain different individuals with different chloroplast types. In addition, single individuals with the 440 bp may not be homoplasmic with respect to the 440 bp allele since the "0" allele is invisible. The specificity of the "0" allele can not be determined as it was present both in *B. oleracea* representatives and in the two oilseed rape varieties know to harbour *B. rapa* chloroplasts and there may be several distinct causes giving the "0" allele.

Since the inheritance was regular under conditions allowing only BC_1 production, spontaneous transfer does not seem very likely. This is good news for developers of transplastomic oilseed rape, but it is important to remember that this approach is not a guarantee for containment of transgenes. Reboud and Zeyl (1994) stated that small sample sizes may have biased the prevailing view of organelle inheritance by under-estimating the occurrence of low-frequency paternal transmission of organelles. We examined 122 BC_1s in total in the present experiment. In another study, we investigated the paternity of progenies produced by F_1-hybrids coexisting with *B. napus* and *B. rapa* in three different proportion and three different densities. The frequency of BC_1s

produced with *B. rapa* as recurrent parent ranged within 0.6-7.8% (Johannessen et al., Paper III). Under conditions with occasional paternal inheritance, these BC_1s would not pose a problem to the environment since they would reduce the abundance of genetically modified plastids in the environment. The paternity of progenies, made on *B. rapa* coexisting with *B. napus* and F_1-hybrids in different proportions and densities, was determined by Pert et al. (2002). It showed that 0-1.5% of the progenies had F_1-hybrids as father except for the 0:1:35 (*B. napus*:*B. rapa*:F_1-hybrids) proportion where 5-6% of the progeny was sired by F_1-hybrids. Thus, the conditions need to be extreme to give a high production of BC_1s who potentially could have received engineered plastids by irregular inheritance. These BC_1s could pose a problem to the environment since they would increase the abundance of genetically modified plastids in the environment. In conclusion, when F_1-hybrids are coexisting with *B. napus* and *B. rapa*, BC_1s with *B. rapa* as the recurrent parent will be produced with both F_1-hybrids and *B. rapa* as mother. It will happen in a frequency of 0-7.8% depending on the environmental conditions.

In conclusion, from the specific markers developed we could infer that the inheritance of plastids was maternal in all cases in the material investigated. Even though the chance that a transgene incorporated into the plastids would be inherited in an irregular manner is small, the traits selected should still be chosen with caution because plants are dynamic and mechanisms may change; they do occasionally as was seen in the *F. pratensis*-*L. perenne*-model. Deviation from the usual may indicate that the organelle exclusion and inactivation mechanisms are not absolute.

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Table 1 The total number of developed seeds and the number of progenies investigated for chloroplast inheritance within each cross between B. rapa *and F_1-hybrids (*B. napus *(♀) x* B. rapa*).*

Parents		Total number	Number of progenies
♀	♂	of seeds	investigated
F_1-hybrid 1	*B. rapa* 1	1	0
F_1-hybrid 2	*B. rapa* 2	18	10
F_1-hybrid 3	*B. rapa* 3	0	0
F_1-hybrid 4	*B. rapa* 4	14	10
F_1-hybrid 5	*B. rapa* 5	87	10
F_1-hybrid 6	*B. rapa* 6	45	10
F_1-hybrid 7	*B. rapa* 7	27	9
F_1-hybrid 8	*B. rapa* 8	0	0
F_1-hybrid 9	*B. rapa* 9	7	6
F_1-hybrid 10	*B. rapa* 10	16	10
∑			65
B. rapa 11	F_1-hybrid 11	46	8
B. rapa 12	F_1-hybrid 12	2	0
B. rapa 13	F_1-hybrid 13	8	2
B. rapa 14	F_1-hybrid 14	21	8
B. rapa 15	F_1-hybrid 15	14	7
B. rapa 16	F_1-hybrid 16	6	3
B. rapa 17	F_1-hybrid 17	87	9
B. rapa 18	F_1-hybrid 18	17	9
B. rapa 19	F_1-hybrid 19	45	6
B. rapa 20	F_1-hybrid 20	18	5
∑			57

*Table 2 Allele frequencies ('a'-'d' primer pair, digested with a*Taq *I) among spring and winter types of oilseed rape, released at different times, with or without chloroplasts known to originate from* B. rapa*, as well as allele frequencies in* B. rapa *and F_1-hybrids.*

	Allele		
Plant material (type, year of release, origin of chloroplasts)	440 bp	455 bp	"0"
Matador (winter, 1949, ?)	1/5		4/5
Vestal (winter, 1956, ?)			5/5
Victor (winter, 1963, ?)	3/5		2/5
Express (winter, 1993, ?)		5/5	
Capitol (winter, 1996, ?)		5/5	
Artus (winter, 1998, ?)	5/5		
Line (spring, 1978, ?)			5/5
Drakkar (spring, 1987, ?)		5/5	
Isuzu Natane (intermediate, ?, *B. rapa*)			5/5
Petranova (spring, ?, *B. rapa*)	3/5		2/5
B. oleracea (-,-,-)			3/3
B. rapa (-, -, -)	10/10		
F_1-hybrids (Capitol (♀) x *B. rapa*) (-, -, -)		10/10	

Paper III

Competition affects the production of first backcross offspring on F_1-hybrids, *Brassica napus* x *B. rapa*

(Submitted to *Euphytica*)

Competition affects the production of first backcross offspring on F_1-hybrids, *Brassica napus* x *B. rapa*

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Keywords: density, field trial, glufosinate-ammonium tolerance, Inter-SSR, proportion, transplastomic

Abstract

Transplastomic F_1-hybrids may arise in fields with transplastomic oilseed rape where weedy *B*. *rapa* occurs as a weed. Spilled seeds, including transplastomic F_1-hybrids, may germinate, which creates an opportunity for production of transplastomic BC_1s with *B*. *rapa* as father (BC_{1r}). Field trials were made with three different proportions of *B*. *napus*, *B*. *rapa* and F_1-hybrids and three different densities. Contrary to most studies on effects of plant competition on introgression between oilseed rape and *B*. *rapa*, this study was focused on offspring produced on F_1-hybrids, where the F_1-hybrids had oilseed rape as maternal parent. We estimated the BC_{1r} production in all combinations of proportion and density. At the proportion with the highest abundance of F_1-hybrids the entire paternity was assessed. There was a significant density effect on the production of BC_{1r}s but the effect differed among proportions. Both the highest and lowest frequencies of BC_{1r}s were obtained at high plant density. Neither the proportion nor density affected the number of BC_{1r}s per square-meter significantly. Biomass components decreased significantly from low to intermediate density, whereas a further increase in density only affected the thousand-kernel weight significantly. When the total paternity was assessed, *B*. *napus* was the predominant father.

We conclude that introgression of transgenes from transplastomic oilseed rape to *B*. *rapa* is most likely at current field densities of *B*. *napus* and when *B*. *rapa* is an abundant weed.

Introduction

When investigated, intraspecific (*B. napus*) (Erickson & Kemble, 1990) and interspecific (*B. rapa* (♀) x *B. napus*) (Scott & Wilkinson, 1999) plastid inheritance has been strictly maternal. Fields of transplastomic oilseed rape with *B. rapa* (~ *B. campestris*) as a weed or with *B. rapa* stands in close proximity are therefore not likely to produce transplastomic F_1-hybrids on *B. rapa*. However, *B. rapa* may sire progenies made on transplastomic *B. napus* (Jørgensen & Andersen, 1994; Johannessen et al., Paper I) and these F_1-hybrids will be transplastomic. At and during harvest of transplastomic oilseed rape a proportion of the seeds will be spilled (Tolstrup et al., 2003) including transplastomic F_1-hybrids. The frequency of F_1-hybrids among the spilled seeds depends on the environmental conditions under which the oilseed rape was cultivated. The plant density and especially the abundance of *B. rapa* are important factors (Johannessen et al., Paper I). Crop rotation and weed management may delay coexistence between the F_1-hybrids and *B. rapa*/*B. napus*, but F_1-hybrids may have seed-dormancy (Landbo & Jørgensen, 1997) and therefore reside in the soil for several years together with seeds from the *B. rapa* population that sired them.

Favourable conditions for F_1-hybrid and *B. rapa* seed germination may arise the next time oilseed rape is cultivated in the area and enables production of BC_{1r}s (first backcross generation with *B. rapa* as the recurrent paternal parent). Spontaneously produced backcross plants have previously been detected in experimental plots and in nature (Hansen et al., 2001; Hansen et al., 2003; Jørgensen et al., 1996; Mikkelsen et al., 1996a).

Investigation of chloroplast inheritance in reciprocal crosses between *B. rapa* and F_1-hybrids, showed an apparently strictly maternal inheritance (Johannessen et al., Paper II). However, with *B. rapa* as the recurrent paternal parent, transplastomic BC_1s (BC_{1r}s) may be produced, and the transgene be further introgressed into *B. rapa*. An estimate of the extent of BC_1s produced on F_1-hybrids with *B. rapa* as father is of interest when considering the risks of cultivating transplastomic oilseed rape.

We investigated the following:

1. Is the BC_{1r} production on F_1-hybrids influenced by different proportions and densities between *B. napus*, *B. rapa* and F_1-hybrids?
2. How is the paternity of F_1-hybrid offspring at the proportion with the highest abundance of F_1-hybrids (1:1:1, *B. napus*:*B. rapa*:F_1-hybrids)?
3. Do different proportions and densities change the vegetative and reproductive fitness of F_1-hybrids?

We estimated the BC_{1r} production on F_1-hybrids (not transplastomic) cultivated together with *B. rapa* and *B. napus* at three different proportions and three different densities. The BC_{1r}s were identified by tolerance against the herbicide BASTA®, the BC_{1n}s (*B. napus* as the recurrent parent) by their genetic fingerprint; progenies harvested on F_1-hybrids with Artus (oilseed rape variety) specific DNA-markers were BC_{1n}s, and F_2 offspring (self pollinated F_1-hybrids) were those remaining that were not identified as BC_{1r} and BC_{1n}. Fitness of hybrids is often strongly dependent on the environment (Arnold, 1997) thus, to reveal the effect of plant competition on vegetative and reproductive fitness of F_1-hybrids, several fitness parameters were determined.

Methods and materials

Plant material

F_1-hybrids (AAC, 2*n* = 29) were obtained by controlled crosses between the winter variety of oilseed rape Capitol ("Cargill", France, *B. napus* genome constitution AACC, 2*n* = 38) and weedy *B. rapa* (AA, 2*n* = 20) consisting of a mix of four wild populations collected in southeast Zealand. For details about the F_1-hybrid production, see Johannessen et al. (Paper I). The winter oilseed rape Artus ("Norddeutsche Pflazenzucht", Germany) was used as the *B. napus* variety in the field trial. Employment of two different oilseed rape varieties eased the development of specific markers and it may resemble agricultural practice, as new varieties probably would be developed in the time between subsequent oilseed rape cultivation due to crop rotation.

BC_5 plants with transgenic tolerance against the herbicide glufosinate-ammonium (BASTA®) were used as *B. rapa*. The herbicide tolerance was used as a selectable marker to assess the paternity of offspring on F_1-hybrids sired by *B. rapa*. Figure 1 outlines crossing details for the BC_5 production. Previous investigations (Mikkelsen et al., 1996a) had shown that the BC_1 generation used in our BC_5 production had a pollen fertility of > 95% and 20-21 chromosomes. Moreover, in the BC_3 generation the plants produced at least as many seeds as pure *B. rapa* and thus probably were very *B. rapa* like, despite the fact that a portion of their genome was derived from *B. napus* (at least the transgene encoding BASTA® tolerance) (Snow et al., 1999). On average 1/64 of the A-genome from *B. napus* would be represented in the BC_5 generation, however the large homology between the A-genomes of *B. napus* and *B. rapa* (Lydiate et al., 1993) further supports that the BC_5 generation was rather *B. rapa* like. However, as noticed in Snow et al. (1999) the transgenic progeny was always used as maternal plants, and therefore the BC_3 generation possessed cytoplasmic DNA from *B. napus*. Origin of cytoplasm from *B. rapa* was therefore ensured by using *B. rapa* as the maternal parent when BC_4 and BC_5 seeds were produced. Flowers were emasculated on maternal plants and bumblebees from mini-beehives made the pollinations. Plants derived from the BC_5 seeds are referred to as *B. rapa*. Seeds were germinated following the procedure described in Johannessen et al. (Paper I).

Field trial

The field trial was established early in the summer with seedlings of *B. rapa*, F_1-hybrids and oilseed rape (Artus). Only *B. rapa* plants with tolerance against BASTA® were transplanted, giving a 1:1 mixture of transgenic:non-transgenic *B. rapa* pollen (the BC_3 generation segregated in a 1:1 proportion for tolerance against BASTA® (Snow et al., 1999)). F_1-hybrids and oilseed rape had been vernalized for 10 weeks at 4°C before the transplanting, a treatment not necessary for *B. rapa*. Plots were established with oilseed rape, *B. rapa* and F_1-hybrids in a 3:1:1 (*B. napus*:*B. rapa*:F_1-hybrids) proportion corresponding to a realistic scenario in some Danish agricultural areas, in a 3:3:1 proportion corresponding to a scenario with a higher weed incidence and in a 1:1:1 proportion as a worst-case scenario. The recommended field density of oilseed rape is 60-90 plants/m^2 (Danish Agricultural Advisory Service, Århus, Denmark). We chose densities of 16 plants/m^2 (25 cm between plants) 44.5 plants/m^2 (15 cm between plants) and 100 plants/m^2 (10 cm between plants), following the experimental setup in Johannessen et al. (Paper I), giving nine plots in all. The plots were separated by 10 m of winter wheat. Plants were planted with equal distance to comply the densities and in specific patterns to ensure that the proportions were accomplished. The plots were

quadratic and included 150 *B. napus*, 50 *B. rapa* and 50 F_1-hybrids at the 3:1:1 proportion, 150 *B. napus*, 150 *B. rapa* and 50 F_1-hybrids at the 3:3:1 proportion and 50 of each plant type at the 1:1:1 proportion. Consequently the plots were of different size.

Plants were sprayed against pollen beetles (*Meligethes aeneus*) through the flowering period. After eleven weeks, the seeds on F_1-hybrids were mature and plants inside the two outermost rows surrounding the plots were harvested. The plants were cut just above the ground and left to dry in open paper-bags. Ten uniformly distributed F_1-hybrid mother plants were selected per density and proportion, giving 90 mother plants in all, and for each the number of pods, the yield (total seed weight), the seed-number and the vegetative dry biomass were determined.

Identification of fathers

At the 3:1:1 and 3:3:1 proportion at least 45 seeds per F_1-hybrid mother plant were germinated if possible (occasionally reduced due to low seed production, low seed viability or seed dormancy). With regard to the paternity, we examined whether or not *B. rapa* was the father of the offspring. Spraying with 0.5% (v/v) BASTA® was used to identify BC_1s with *B. rapa* as the father (BC_{1r}s). When sired by the hemizygous transgenic *B. rapa*, half are expected to carry the *bar* gene and thus be tolerant to BASTA®. The total number of offspring plants sired by *B. rapa* were then calculated as the double of the number of BASTA® tolerant plants.

As to the remaining proportion (1:1:1) up to 45 seeds from each F_1-hybrid mother plant were germinated, giving about 1350 progenies for total analysis of paternity (i.e. oilseed rape, *B. rapa* or F_1-hybrids).

An Artus (oilseed rape variety) specific marker was developed to reveal BC_{1n} offspring. For this purpose DNA extraction and Inter-SSR-PCR was made according to the procedure described by Johannessen et al. (Paper I). PCR was made with the degenerate primer 888 (BDB-[CA_7]) (Charters et al., 1996), tagged with a 700nm or 800nm fluorescence. After the PCR, 3.5 µl 700nm product, 6.5 µl 800nm product and 5 µl formamide loading buffer (bromphenol blue xylen cyanol dye solution, Sigma-Aldrich) were mixed, allowing separation on two channels per electrophoresis. The samples were heated at 96°C for 5 minutes, quickly cooled on ice, and then loaded on a 4% polyacrylamide gel. Electrophoresis was made on a LI-COR®, dNA Sequencer, 4200 and visualized by BaseImageIR v. 4.1. An estimate of the specificity, frequency and inheritance pattern of the marker was obtained by analyses of 100 individuals of each of *B. rapa*, F_1-hybrids, Capitol, Artus, and self-pollinated individuals of Artus (isolated in pollen-tight bags).

Offspring sired by *B. rapa* was identified by application of 1% (v/v) BASTA® on an area of 1-2 cm^2 on a single leaf. Tolerant offspring was retested to confirm the results. The total number of offspring plants sired by *B. rapa* was calculated as the double of the number of BASTA® tolerant plants.

The total number of offspring minus the offspring sired by Artus or *B. rapa* was interpreted as self-pollinated (F_2).

Data analyses

We have formulated a hypothesis as follows:

The composition (proportion) and density of plants are environmental factors changing the competitive conditions. Changes in the competitive conditions are reflected in the vegetative fitness, which has an effect on the reproductive fitness. Both vegetative and reproductive fitness affect pollination/fertilization; therefore these environmental factors have an effect on the frequency of BC_{1r}s per mother plant and the number of BC_{1r}s per square-meter, as well as the sirering success of potential fathers (as analyzed at the 1:1:1 proportion).

Data were subjected to analysis of variance (ANOVA, software: SAS version 8.2, SAS Institute Inc., Cary, NC, USA). All data were square root transformed, as it improved the distribution of the residuals. The analyses were made with the frequency of BC_{1r}s per mother plant and the number of BC_{1r}s per square-meter as the dependent variables, as well as the pod-number, the yield, the seed-number, the vegetative dry biomass and the thousand-kernel weight since these biomass components express the fitness of the F_1-hybrid mother-plants. The analyses involved the proportion and density of plants in the field as factors. With the frequency of BC_{1r}s per mother plant and the number of BC_{1r}s per square-meter as dependent variables the pod-number, yield, seed-number, vegetative dry biomass and the thousand-kernel weight were included in the analyses as covariates. Additional analyses were made with the frequency of fathers (*B. napus*, *B. rapa* and F_1-hybrids) at the 1:1:1 proportion as the dependent variable with the paternity and density as factors.

Results

B. rapa sired offspring within all combinations of proportion and density (Table 1), but not within all mother plants. The ANOVA showed no significant effect of density, proportion and their interaction with the frequency of BC_{1r}s as the dependent variable (see Table 2). When the vegetative dry biomass was included in the model as covariate the model made a significant difference when tested against the reduced model (without the vegetative dry biomass) and the improved explanation of the data by the vegetative dry biomass revealed a significant density effect. The density effect was however not unambiguous as the frequency of BC_{1r}s increased at the 3:3:1 proportion and oppositely decreased at the 3:1:1 and 1:1:1 proportion when the density was increased (see Table 1). T-tests revealed significant effects of density on the frequency of BC_{1r}s at each proportion and significant effects of proportion on the frequency of BC_{1r}s at each density (results not shown).

From an agricultural viewpoint, the BC_{1r} production per area unit is an interesting quantity, because it takes plant density and seed-number per mother plant into account. There was no significant effect of density, proportion and their interaction with the number of BC_{1r}s per square-meter as the dependent variable, and the covariates did not improve the explanation of the data significantly. T-tests revealed significant effects of density on the number of F_1-hybrids per square-meter at each proportion and significant effects of each proportion on the number of F_1-hybrids per square-meter at each density (results not shown).

There was a significant decrease in most biomass components per F_1-hybrid mother plant when density was increased from low to intermediate (Table 3). Further increase in density only affected the thousand-kernel weight significantly, which increased.

ANOVAs revealed a significant density effect, whereas the effect of proportion was non-significant for all dependent variables except the thousand-kernel weight (Table 4).

The DNA-marker used for identification of offspring sired by Artus at the 1:1:1 proportion was Artus specific and monomorph. Additionally, the self-pollinated individuals of Artus all displayed the marker, thus the marker was interpreted as homozygous in Artus and allowed us to detect all offspring on F_1-hybrids sired by Artus.

At the 1:1:1 proportion *B. napus* was the most frequent father, followed by F_1-hybrids and finally *B. rapa* (Table 1). ANOVAs revealed an almost significant density effect, a significantly different distribution of fathers between the three possible kinds (paternity), plus a significant effect of the interaction between density and paternity. Thus the relative sirering success of the three possible fathers depended on the density.

Discussion

The abundance of BC_{1r}s

The frequency of BC_{1r}s produced on F_1-hybrids was density dependent. When the density intensified the competition, the frequencies differed among proportions, revealing that the composition of the population was important. The highest frequency of BC_{1r}s was obtained at high density at the 3:3:1 proportion where the fitness of mother plants was the lowest observed for most biomass components including the total biomass. Since the total biomass is regulated rather precisely and is expected to be the best measure of competition (Begon et al., 1990), the F_1-hybrids was thus exposed to the most intense vegetative competition at high density, with the highest abundance of *B. rapa* and the lowest abundance of themselves. Thus intense competition seems to favour backcrossing. The lowest frequencies of BC_{1r}s were obtained as well at high density, but at the two other proportions, thus the proportion was an important determinant for the outcome at this specific density. When the density of plants was increased, so were perhaps the density of flowers and the density of pollen. Consequently closer neighbours maybe encouraged insects to change more frequently between plants and the pollen cloud (wind pollination) maybe to a larger extent consisted of pollen proportional to the plant proportion. The pollen was therefore probably more easily transferred at high density, but the dependence on neighbouring pollen or on the composition of the pollen cloud is not obvious. The reason why a high abundance of *B. rapa* (the 3:3:1 proportion compared with the 3:1:1 proportion) should favour the production of BC_{1r}s is probably resulting from a relative increase in *B. rapa* pollen. Both the low abundance of F_1-hybrids (the 3:3:1 proportion compared with the 1:1:1 proportion) and the intense vegetative competition, which probably affected the reproductive fitness (i.e. fewer branches, fewer racemes, fewer flowers, less pollen), most likely reduced the contribution of F_1-hybrid pollen.

Effects of competition on seed weight and seed set

The thousand-kernel weight of *B. napus* mother plants was unaffected by changes in density (Johannessen et al., Paper I), however for F_1-hybrid mother plants the same parameter was in most cases significantly higher when the density was increased. The feature may therefore be associated to the *B. rapa* genome of the F_1-hybrids. Additionally, the duration of seed maturation may be essential for the seed weight as accelerated maturation of *B. napus* seeds induced by heat treatment has been shown to drastically decrease the seed weight (Aksouh et al., 2001). Therefore, since the other

biomass components decreased when density was increased the F_1-hybrid mother plants perhaps reached the final development stages earlier at high density, which left more time for maturation and seed filling, giving heavier seeds.

Whether the F_1-hybrids were strong or weak competitors depended on the density and were revealed by the total biomass. At low density for example *B. napus* and *B. rapa* were weaker competitors to the F_1-hybrid, than the F_1-hybrid was to itself, whereas it was reverse at high density. It is not possible to say how the competitive interactions affected *B. napus* and *B. rapa* since their biomass components were not assessed.

In a field trial with F_1-hybrids (*var*. Drakkar, spring type as father) coexisting with *B. napus* (*var*. Drakkar spring type) and *B. rapa* in different proportions and the same densities as we used, F_1-hybrids produced many more seeds in mixtures than in pure stands or in mixtures converging pure stands (Hauser et al., 2003). Except for the low density at the 3:3:1 proportion this was opposite in our experiment. Winter types of *B. napus* were used for the F_1-hybrid production (*var*. Capitol) and as the oilseed rape variety (*var*. Artus) in the field trial. Winter types are larger and higher yielding than spring types, and therefore likely stronger competitors, which perhaps affected the competitive interactions. Furthermore the interaction between two different oilseed rape varieties (*B. napus* and the F_1-hybrids) perhaps also affected the competition.

The abundance of BC_{1r}s per square-meter

The number of BC_{1r}s per square-meter (based on the exact seed set and the frequency of F_1-hybrids per mother plant) changed with the combination of proportion and density. There were no clear tendencies when the density was changed, however as to the proportion the number of BC_{1r}s per square-meter seemed to be low when the proportion of *B. rapa* was low (3:1:1) and comparably high when the abundance of F_1-hybrids was high (1:1:1). There were no significant effects of density and proportion. The number of BC_{1r}s per square-meter was highest at low density (least intense competition) and the 1:1:1 proportion, resulting from large seed set on mother plants and the high abundance of F_1-hybrids at this proportion.

The lowest number of BC_{1r}s per square-meter was obtained at intermediate density and the 3:1:1 proportion. This resulted from the identification of most BC_{1r}s on one mother plant with a low seed set. The low seed set on this mother plant could have been the result of low reproductive fitness, however it was probably caused by a low vegetative fitness of the plant since it had a low dry biomass, and thus few branches and flowers.

In controlled crosses 9.8 seeds were obtained per pollination when F_1-hybrids (*B. napus* (♀) x *B. rapa*) were produced and only 0.7 seeds per pollination when BC_{1r}s (F_1-hybrids (♀) x *B. rapa*) were produced (Mikkelsen et al., 1996b). Under a range of environmental conditions the spontaneous F_1-hybrid production was 0.4-5.3% corresponding to 71-1614 F_1-hybrids per square-meter (Johannessen et al., Paper I) and the spontaneous BC_{1r} production was 0.6-7.8% corresponding to 289-1962 BC_{1r}s per square-meter (present paper). This illustrates that what seems to be a barrier to introgression under controlled conditions may not be a barrier at all under natural conditions. This, as often, underlines the need for field trials before drawing final conclusions about environmental consequences.

The paternity at the 1:1:1 proportion

The density affected the competitive interactions so that each of the three paternal genotypes obtained their maximum paternity-frequency at different densities. We expected the 1:1:1 proportion to produce most BC_{1r}s as the abundance of *B. napus* was low, and thereby could improve the vegetative and reproductive conditions for both *B. rapa* and F_1-hybrids. The BC_{1r} productions per square-meter were higher at this proportion, but the frequencies of BC_{1r}s were not. The abundance of F_1-hybrids and thus their pollen contribution would be expected to be the largest at this proportion and thereby increase self-pollination. The chromosome complement of self-pollinated F_1-hybrids may, depending on the genome constitution of the gametes (n = A + 0-9C), be identical to BC_{1r}s, however, C-chromosomes can be transferred at comparatively high frequencies to the first backcross generation (Mikkelsen et al., 1996b). Thus, the overall introgression depends on the abundance of BC_{1r}s as well as offspring from self-pollinated F_1-hybrids, taking their genome constitution into consideration. However we did not analyze the genomic status of the F_1-hybrid offspring.

The vast majority of offspring was sired by *B. napus*. A study showed that in *B. rapa* styles there was no effect of pollen being of *B. rapa* or *B. napus* type, whereas heterospecific pollen had a lower fitness on *B. napus* styles (Hauser et al., 1997). One could speculate that *B. rapa* and F_1-hybrid pollen had lower fitness on F_1-hybrid styles than *B. napus* pollen leading to the apparent preference for *B. napus* pollen. This seems reasonable due to the highly variable and sometimes rather low pollen fertility of F_1-hybrids (Jørgensen et al., 1994; U, 1935). In another study investigation of the pollen production and viability revealed that *B. napus*, *B. rapa* and F_1-hybrids did not differ much in total pollen production, but that pollen from F_1-hybrids was much less viable than *B. napus* and *B. rapa* pollen (Pertl et al., 2002). In that study it was further argued that the competitiveness of pollen from F_1-hybrids probably was decreased because of the aneuploid genome constitution (A + 0-9C).

Other post- and prefertilization barriers could have affected the paternity distribution. In *B. napus* and *B. rapa* there is also reduced zygote survival in hetero versus conspecific zygotes (Hauser et al., 1997) probably as a result of genomic incompatibility. If the zygote survival was reduced when either of the potential fathers pollinated F_1-hybrids these fathers may have been more frequent pollinators than expressed by the distribution of fathers among the offspring. The frequency of *B. rapa* as father was perhaps affected by the overlap of gene pools, and thereby incompatibility alleles, between the F_1-hybrid and the *B. rapa* populations. Their fathers were obtained from the same *B. rapa* population, thus sporophytic self-incompatibility (Bateman, 1955) may have acted. Selfing of F_1-hybrids was investigated by Mikkelsen (1996), who revealed a low pod and seed set explained by incompatibility reactions or low F_1-hybrid fertility. If incompatibility reactions act within F_1-hybrid and *B. rapa* populations they are also likely to act between them when gene pools are partly shared. However, the situation resembles a natural scenario as F_1-hybrids in the field will derive from a local *B. rapa* population, which again will be the most likely *B. rapa* pollinator when the F_1-hybrid seeds are germinating subsequently.

Seed dormancy is a trait associated with *B. rapa* and also of its BC_1 generation (Landbo et al., 1997) and may explain why the seed germination of offspring differed among mother plants. However, the germination frequencies were not registered. As discussed in Pertl et al., (2002) the non-germinated seeds produced on *B. rapa* which had coexisted with *B. napus* (spring type) and F_1-hybrids (*B. rapa* (♀) x *B. napus* (♂)) were probably pure *B. rapa* or BC_{1r}s, which for some reason was not affected by the dormancy breaking treatment. Similarly, the non-germinated seeds in our experiment perhaps were *B. rapa*-

like BC_{1r}s or F_2s. If this is true, the paternity estimates are biased with too many offspring sired by *B*. *napus*, thus underestimating the introgression into *B*. *rapa*.

Hybridization (Johannessen et al., Paper I) and back crossing (present paper) per mother plant was most pronounced at high density with a high proportion of *B. rapa*, which suggests that that introgression of transgenes into *B*. *rapa* is most likely at field densities and when *B*. *rapa* is an abundant weed. This is contrary to the results from the alternative introgression route with transgenes integrated in the nuclear DNA. Here, with *B*. *rapa* functioning as the maternal parent, it was shown that hybridization was most pronounced at low plant density, which suggested that introgression is most likely at set-aside land, ruderal sites and fields where Brassicas are weedy in other crops (Pertl et al., 2002).

The number of F_1-hybrids (Johannessen et al., Paper I) and BC_{1r}s (present paper) per square-meter were lowest when the proportion of *B. rapa* was lowest. The density also affected the numbers but there were no clear tendencies. Therefore efficient weed control in the field and adjacent areas is an important determinant for the introgression process with *B*. *rapa* as the recurrent paternal parent; the only transgene escape route when cultivating transplastomic oilseed rape.

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Maternal parent		Paternal parent
Thirteen *B. rapa* plants Weedy populations containing Only B. rapa specific markers (Hansen et al., 2001) Collections: Bc29-3 Bc30-1	x	**One-hundred-and-fifty BC_3 plants (Snow et al., 1999)** Selected as tolerant to glufosinate-ammonium 1-10 1-18 1-13 9-15
	↓	
	BC_4 Segregating for glufosinate-ammonium tolerance	
Eight *B. rapa* plants Seeds produced from a mix of four wild populations collected in southeast Zealand	**x**	**One-hundred BC_4 plants** Selected as tolerant to glufosinate-ammonium tolerance
	↓	
	BC5 Segregating for glufosinate-ammonium tolerance	

Figure 1 Crossing scheme for obtaining BC_5 seeds.

*Table 1 The average production of offspring on F_1-hybrids (*B. napus *(♀) x* B. rapa*) with* B. rapa *as father given as the frequency and the number per square-meter at three different proportions (3:3:1, 3:1:1 and 1:1:1) and three different densities (low, intermediate and high). The complete paternity (*B. napus, B. rapa *or F_1-hybrids) of offspring produced on F_1-hybrids given as the frequency at the 1:1:1 proportion and three different densities.*

Proportion (*B. napus*: *B. rapa*: F_1-hybrids)	Father	Density					
		Low		Intermediate		High	
		Frequency per mother plant	Number per m^2	Frequency per mother plant	Number per m^2	Frequency per mother plant	Number per m^2
3:3:1	*B. rapa*	4.1 %	511	6.1 %	491	7.8 %	1431
3:1:1	*B. rapa*	4.3 %	559	3.7 %	289	0.6 %	520
1:1:1	*B. rapa*	4.0 %	1962	2.8 %	1131	0.9 %	1481
	B. napus	87.1 %	-	93.5 %	-	80.1 %	-
	F_1-hybrid	8.9 %	-	3.8 %	-	19 %	-

Table 2 ANOVA with frequency of BC_{1r}s, BC_{1r}s per square-meter and frequency of fathers (1:1:1 proportion) as the dependent variables.

Dependent variable	Test of effect of	df	F	P
BC_{1r} (frequency)	Density	2	2.36	0.1013
	Proportion	2	2.17	0.1212
	Density*Proportion	4	0.85	0.5005
	Density	2	6.15	0.0032
	Proportion	2	1.95	0.1489
	Vegetative dry biomass	1	7.25	0.0086
BC_{1r} (per square-meter)	Density	2	1.37	0.2606
	Proportion	2	0.91	0.4061
	Density*Proportion	4	0.81	0.5222
Father (frequency)	Density	2	2.06	0.1388
	Paternity	2	397.07	<0.0001
	Density	2	2.95	0.0580
	Paternity	2	568.75	<0.0001
	Paternity*Density	4	10.19	<0.0001

*Table 3 Mean value of biomass components (pod-number, yield, thousand-kernel weight, vegetative dry biomass, seed–number, seeds per pod and total biomass) per F_1-hybrid mother plant (*B. napus *(♀) x* B. rapa*) for each combination of density (low, intermediate and high) and proportion (3:3:1, 3:1:1 and 1:1:1) (± 95% level of confidence).*

Proportion (*B. napus*: *B. rapa*: F_1-hybrids)	Biomass component	Density				
		Low		Intermediate		High
3:3:1	Pod-number	485 (±131)	>>	176 (±86)	=	94 (±38)
	Yield (g)	6.4 (±2.8)	>>	1.1 (±0.7)	=	0.7 (±0.3)
	Thousand-kernel weight (g)	1.7 (±0.3)	=	1.9 (±0.3)	<	2.5 (±0.3)
	Dry biomass (g)	38.7 (±8.0)	>>	12.2 (±6.1)	=	6.0 (±2.4)
	Seed-number	4057 (±1685)	>>	502 (±209)	=	299 (±139)
	Seeds per pod	9.5 (±5.0)	>	2.9 (±0.5)	=	3.0 (±1.2)
	Total biomass (g)	45.1 (±10.3)	>>	13.3 (±6.7)	=	6.7 (±2.7)
3:1:1	Pod-number	616 (±161)	>>	135 (±39)	=	111 (±45)
	Yield (g)	4.3 (±1.3)	>>	1.2 (±0.7)	=	1.0 (±0.4)
	Thousand-kernel weight (g)	2.0 (±0.1)	<	2.2 (±0.2)	<	2.8 (±0.4)
	Dry biomass (g)	42.5 (±9.7)	>>	8.7 (±2.7)	=	7.7 (±2.9)
	Seed-number	2205 (±714)	>>	598 (±346)	=	362 (±122)
	Seeds per pod	3.8 (±1.3)	=	4.1 (±1.9)	=	4.2 (±1.7)
	Total biomass (g)	46.8 (±10.3)	>>	9.9 (±3.3)	=	8.7 (±3.2)
1:1:1	Pod-number	497 (±135)	>>	231 (±63)	=	146 (±52)
	Yield (g)	6.0 (±3.1)	>	2.4 (±1.2)	=	1.8 (±0.8)
	Thousand-kernel weight (g)	1.8 (±0.1)	<<	2.2 (±0.2)	<<	2.6 (±1.5)
	Dry biomass (g)	29.4 (±6.2)	>>	16.3 (±4.7)	=	10.5 (±3.1)
	Seed-number	3333 (±1630)	>	1059 (±525)	=	728 (±311)
	Seeds per pod	6.9 (±4.4)	=	4.1 (±1.3)	=	4.5 (±1.2)
	Total biomass (g)	35.4 (±9.2)	>>	18.6 (±5.8)	=	12.3 (±3.7)

Significance of the difference revealed by t-tests: = non-significant, > significant at a 5% level, >> significant at a 1% level.

Table 4 ANOVA with the pod-number, yield, seed number, vegetative dry biomass and thousand-kernel weight as the dependent variable, and density and proportion as factors.

Dependent variable	Test of effect of	df	F	P
Pod-number	Density	2	67.54	<0.0001
	Proportion	2	0.95	0.3920
Yield	Density	2	33.71	<0.0001
	Proportion	2	2.04	0.1358
Seed-number	Density	2	45.45	<0.0001
	Proportion	2	1.89	0.1574
Vegetative dry biomass	Density	2	78.77	<0.0001
	Proportion	2	0.31	0.7377
Thousand-kernel weight	Density	2	30.44	<0.0001
	Proportion	2	4.92	0.0095

Paper IV

Gene introgression and consequences in *Brassica*

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Gene introgression and consequences in *Brassica*

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Abstract

Transgenes may be transferred from genetically modified (GM) crops to the wider environment through crosses with compatible wild or weedy relatives. For oilseed rape (*Brassica napus)* we found extensive transfer of nuclear as well as plastid DNA (cpDNA) to *Brassica rapa* in an environment with poor weed control. Some of the plants with markers from both species were apparently introgressed beyond the stage of the BC_1 generation. In conventionally managed fields with oilseed rape as crop and the wild recipient as a weed, the introgression was insignificant or not detected, so apparently the extent of gene flow from the crop depended on the agricultural management or other environmental effects. Our results also showed that oilseed rape plastids were introgressed to *B. rapa* under field conditions. Field and laboratory experiments revealed that fitness of interspecific F_1 hybrids and backcross plants with *B. rapa* were variable but could be as high as and even higher than the fitness of the parental species.

We present results that show the importance of genotype and environment (i.e. agronomic practice and density/proportion of plant types) in the introgression of oilseed rape genes to *Brassica* and *Raphanus* species. In the light of our findings we discuss the perspectives of releasing genetically modified oilseed rape.

Key words: *Brassica, Raphanus*, interspecific hybridization, introgression, fitness, transgenes, GMO.

Introduction

Flow of transgenes by hybridization between genetically modified crops and wild relatives is not necessarily a risk to the environment. The consequences to natural and cultivated ecosystems depend on a wide range of factors. Therefore the effects are most appropriately addressed by a targeted risk analysis of the transgenic plant in its proper environment. However, baseline knowledge about the extent of gene flow between the crop and related recipients as well as survival of the resulting progeny is always requested in the risk assessment process. In the present paper we present some of our results on introgression of oilseed rape genetic material (*B. napus*, 2n=38, genomes AACC) to related species in the *Brassica* and *Raphanus* genus and fitness analysis of the introgressed plants. In Denmark the wild relatives of oilseed rape *B. rapa* and *R. raphanistrum* are rather common as weeds in agricultural fields. They are mostly found in oilseed rape fields but also in other types of crops where they can form weedy populations together with volunteer oilseed rape. *B. rapa* and *R. raphanistrum* can also occur at ruderal sites but are rarely found in more natural habitats. *B. rapa* and *R. raphanistr*um are likely recipients of oilseed rape genes and therefore quantification of the crop-wild gene flow is relevant. Other less abundant relatives i.e. *B. juncea* might also have potential for spontaneous gene exchange with oilseed rape.

Results and discussion

Gene flow from oilseed rape to *Brassica rapa*

Frequency of F_1 hybrids

B. rapa (2n=20, genomes AA) is one of the parental species of oilseed rape. *B. rapa* is a common annual weed in agricultural fields worldwide in the temperate zone. Outside the field *B. rapa* populations are ephemeral, as seeds will only germinate when the soil is turned. Harberd (1975) reported the spontaneous occurrence of the *B. napus* x *B. rapa* hybrid (*B. x harmsiana*) in oilseed rape fields. Frequencies of F_1 hybrids between oilseed rape and the weedy *B. rapa* have been reported from field experiments and survey of natural populations of the wild species. Jørgensen & Andersen (1994), Jørgensen et al. (1996, 1998), Landbo et al. (1996), Scott & Wilkinson (1998) and Pertl el al. (2002) found hybrid frequencies between 0-69 % of the seeds depending on i.e. parental genotypes, density and proportions of plants and agricultural practice. Generally, *B. rapa* produces more interspecific hybrids than oilseed rape when the two species grow together under natural conditions (Jørgensen & Andersen, 1994; Jørgensen et al., 1998; Hauser et al., 1997).

Frequency of backcrossing in a field experiment

The F_1 hybrids have reduced pollen fertility (Jørgensen & Andersen, 1994; Pertl et al., 2002), but spontaneous backcrossing does take place. *B. rapa* and interspecific hybrids with a transgene providing Basta resistance were sown together in field experiments to assess the extent of backcrossing (Mikkelsen 1996) to the weedy parent. Seed set per pod on interspecific hybrids was low (app. 2.5) compared to seed set on the parental species (16-23). An average of 67% of the plants developed from seeds harvested on 32 interspecific hybrids were herbicide resistant. Among 865 offspring, plants with a *B. rapa*-like morphology were selected for further analysis. A few (0.5%) were almost identical to *B. rapa* (chromosome number 2n=20, high pollen fertility) and set a normal

number of seeds in crosses with genuine *B. rapa* (Mikkelsen et al., 1996). The reciprocal cross *B. rapa* x hybrid was not observed among more than 2000 offspring from 30 *B. rapa* plants.

Introgression between oilseed rape and B. rapa *in a natural environment with poor weed control*

In an organic field of barley and legumes in eastern Denmark some morphologically deviating *Brassica* plants were observed together with weedy oilseed rape and *B. rapa*. At flowering all *Brassica* plants were collected from a 3 m^2 plot. Leaf material from a total of 102 plants was analysed using AFLP markers specific to *B. napus* or *B. rapa*. The development of these species-specific markers was described in Hansen et al., 2001. Among the AFLP markers used in the analysis three were specific to *B. rapa* (one monomorphic and two polymorphic markers; due to the homology between the A genome of *B. rapa* and *B. napus* only few *B. rapa* specific markers were identified) and 21 to oilseed rape (17 monomorphic and four polymorphic markers), 18 of which were positioned on the C-genome. In parallel with the analysis of the natural population, F_1 interspecific hybrids and first and second backcross generations with *B. rapa* were produced and the inheritance of the same markers was studied in offspring from these controlled crosses:. *B. rapa* was the female in the controlled crosses, and as all AFLP markers specific to oilseed rape were transferred to offspring plants, these markers were judged to be nuclear.

We revealed a pronounced introgression in the natural weedy population (Fig. 1, Hansen et al., 2001): 45 plants were introgressed having both oilseed rape and *B. rapa* specific markers. Among the remaining 57 plants, seven had only *B. napus* specific markers and 50 plants had only *B. rapa* markers. Figure 1 gives the distribution of oilseed rape markers in the 102 plants. The monomorphic markers showed that there was only one first generation hybrid (F_1) among the analyzed plants. Infrequency of F_1 hybrids could be due to a small number of oilseed rape plants compared to *B. rapa*. We assume that the natural population had maintained itself since 1987, the last time oilseed rape was cultivated in this field. The proportion of oilseed rape probably decreased since then as *B. rapa* has a better survival over time due to pronounced seed dormancy (Landbo and Jørgensen, 1997). The long existence of the mixed population suggests that some of the plants with DNA markers from both species were advanced generations of introgressed plants**.** This was confirmed by comparing the marker distribution in the natural population with distribution of the very same markers in the BC_1 and BC_2 generation from the controlled crosses (Fig. 1, Hansen et al., 2001). As the marker distribution in BC_2 plants resembled the marker complement in the natural population, we tentatively conclude that the latter was introgressed beyond the BC_1 generation. Progression of introgression was studied in offspring from the parental plants in the field (Hansen et al., 2003), and the marker analysis showed that *B. rapa* most often functioned as the maternal plant in the introgression process, and that the amount of oilseed rape DNA was diminished in the majority of offspring compared to their introgressed maternal plants. However, we found that introgression brought about both incorporation of *B. napus* C-genome DNA into the *B. rapa* genome and exchange of chloroplast DNA producing *B. rapa*-like plants with oilseed rape chloroplasts. The chromosome number was counted in 15 offspring plants from five introgressed females in the natural population. Offspring chromosome numbers were 20-26, and they had from 1 to 12 C-genome specific markers. The presence of plants with C-genome markers and 20 chromosomes suggests

that recombination had taken place between the C-genome of oilseed rape and the A-genome of *B. rapa*.

Spontaneous introgression between oilseed rape and B. rapa *in conventionally managed fields*

The inefficient weed control in the organic field probably accounts for the high frequency of introgressed plants. In accordance with this the frequency of introgressed plants from conventionally grown oilseed rape fields was much lower. In 2450 plants from seeds harvested in eight populations of weedy *B. rapa* found in conventional managed fields we only detected two plants introgressed beyond the F_1-stage and 81 F_1 hybrids. The introgressed plants had more than six *B. napus* specific AFLP markers in addition to the *B. rapa* markers. For the majority of these plants the results were obtained from RAPD and isoenzyme data and only a few hundred plants were analyzed by AFLP using the same markers as in the organic field. As the AFLP provided more markers than the other marker techniques the frequency of introgressed plants might have been underestimated. On the other hand the frequency of introgressed plants is probably overestimated compared to the normal field situation as most of the plants analysed were reared in growth chambers from seeds harvested in *B. rapa* populations. In nature the survival of introgressed plants to the adult and reproductive stage is probably lower. Figure 2 compares the data on introgressed plants from the organic and the conventional fields.

For UK environments a low frequency of transgene dispersal from oilseed rape to *B. rapa* was predicted from findings of a low number of F_1 hybrids germinated from seed harvested on *B. rapa* in natural populations found along rivers (Scott and Wilkinson, 1998). Hybridisation between the true wild *B. rapa* and oilseed rape in UK very much depends on the sympatry of these populations with the crop. Weedy populations of *B. rapa* have now also been found in UK. These populations have a higher frequency of hybridisation than the riverbank populations because they occur as a weed within the oilseed rape crop. AFLP analysis of seed bank material from one of these weedy UK populations has revealed introgression, however, the mature plants studied were not introgressed indicating that fitness of introgressed plants could be substantially decreased (Norris et al., this volume). Our data from the Danish populations demonstrate that introgression between oilseed rape and *B. rapa* can be considerable when the two species are found in long existing mixed populations.

Fitness of introgressed plants

We analysed the fitness of different generations of introgressed plants in field and growth chamber experiments. Hauser et al., 2003 showed that numbers of seed set of F_1 hybrids between *B. napus* and *B. rapa* was dependent on the environmental conditions. The F_1 hybrids could produce more seeds than *B. rapa* (maximum seed set/plant: *B. rapa* 4850; F_1 hybrids 6700), but also fewer seeds dependent on the number and densities of parents and backcross plants in the population. As to the F_2 and BC_1 with *B. rapa*, Hauser et al. (1998) found large variation in fitness with a smaller fraction of these plants being just as fit as *B. rapa* and *B. napus*. In a further advanced backcross programme, BC_1 plants were selected for being *B. rapa*-like and these plants backcrossed to *B. rapa* to obtain BC_3 plants. The reproductive fitness of this BC_3 generation was as great as that for *B. rapa* (Snow et al., 1999). The BC_3 plants segregated 1:1 for a *bar* transgene

providing glyphosinate resistance. There was no difference in the seed set and survival of the GM siblings compared to the non-GM siblings when the herbicide was not applied indicating that a cost associated with the transgene was negligible. The overall conclusion from our fitness data is that in some environments some of the introgressed plants will be as fit as their wild parent and occasionally as fit as the crop. These results support our finding of persistent populations of introgressed plants occurring spontaneously in the agro-ecosystem (Hansen et al., 2001 and 2003).

Transfer of plastid encoded genes from oilseed rape to B. rapa

In *Brassica* like most other angiosperms chloroplasts are not transmitted by the pollen (Corriveau and Coleman, 1988). Therefore, transplastomic oilseed rape where the transgene is engineered into the plastid genome, can only disperse the novel genes through the seed (Daniell et al., 1998; Scott and Wilkinson, 1999). The origin of the chloroplast DNA was analysed in 91 of the plants from the natural population in the organic field mentioned above (Hansen et al., 2003). The analysis was performed by PCR using primers to non-coding regions of the cpDNA (Taberlet et al., 1991). The primers amplify species-specific markers for oilseed rape and for *B. rapa*. The analysis assigned the chloroplast of the plants to either oilseed rape- or *B. rapa*-type and this information was compared to their nuclear AFLP-fingerprint. The cpDNA analysis showed that besides the seven *B. napus*-like plants, one *B. rapa*-like plant and two introgressed plants carried the oilseed rape chloroplast. In a huge and persisting weedy *B. rapa* population in a conventionally managed field, we found that 9 of the 23 plants analyzed carried oilseed rape chloroplasts. The AFLP analysis of these plants failed to detect any oilseed rape specific nuclear markers but an AFLP marker segregating together with the *B. rapa* cytoplasm was also missing, supporting that chloroplast introgression had taken place in these plants. Our results from these long-lasting populations of oilseed rape and *B. rapa* indicate that transgenes positioned in the chloroplast DNA of *B. napus* will be captured by *B. rapa*. If oilseed rape and subsequently the F_1 hybrid are females in the crosses, fully fertile *B. rapa*-like plants with 20 chromosomes and oilseed rape chloroplasts can be produced after just two generations (Mikkelsen et al., 1996). This could be a pathway for production of transplastomic *B. rapa.* Scott and Wilkinson (1999) analysed the chloroplast inheritance in 47 F_1 hybrids harvested on wild *B. rapa* and sired by oilseed rape. They only found plants with a *B. rapa* cytoplasm and concluded that transgene introgression from transplastomics would occur extremely rarely in populations of *B. rapa* found along riverbanks in UK.

Gene flow from oilseed rape to *B. juncea*

In northern Europe *B. juncea* (2n=36, genomes AABB) is rarely cultivated but it may occasionally occur as a weed or as a ruderal plant. Spontaneous hybridisation between oilseed rape and *B. juncea* has been reported (Frello et al., 1995, Jørgensen et al., 1998). Depending on the proportions between the parental species up to 3% of the offspring harvested on *B. juncea* were hybrids. Production of hybrids with *B. napus* as female was less successful (Jørgensen et al., 1998). Pollen fertility of the hybrids was rather low, 0-28%. In a study of marker transfer from oilseed rape to the first backcross generation with *B. juncea*, 20 *B. napus* specific RAPD markers were all transferred and most of them in the expected frequencies (Frello et al., 1995).

Gene flow from oilseed rape to *Raphanus raphanistrum*

R. raphanistrum (2n=18, genomes RR) is a weed in agricultural fields and occurs also as a ruderal plant. In separate pollen tight growth cabinets plants from wild populations of *Raphanus raphanistrum* were mixed with a male sterile oilseed rape line and bumble bees were used as pollinators. A Danish, a Swiss and a French population of *R. raphanistrum* were used as paternal parents and seeds were harvested on the male sterile oilseed rape. The offspring was typed by species specific ISSR (inter simple sequence repeats) markers and morphology. The results showed large differences in hybridization potential between the different populations of *R. raphanistrum*, the Danish population being the least likely father of F_1 hybrids as only 0,02 F_1 hybrids were produced per pod compared to the Swiss population that produced many times more hybrids per pod (0,64). Such difference in the hybridization potential among populations or varieties of *Brassicaceae* may be common (i.e. Gueritaine et al., 2003) and may influence the frequency of gene introgression and result in regional variations in risk associated with GM oilseed. Even though F_1 hybrids between oilseed rape and *R. raphanistrum* can be formed the recombination of oilseed rape genetic material into the genome of *R. raphanistrum* seems to be difficult. Chevre et al. 2003 (see this volume) did not observe genomic recombination despite recurrent backcrosses to the wild parent.

Effects of growing genetically modified oilseed rape

Our gene flow analysis is based on DNA markers of the oilseed rape nucleus and plastids. Most of these markers are supposed to be selectively neutral. There is no reason to believe that transgenes will be introgressed differently than these endogenous genes. However, the rate of transgene transfer may be increased due to selection in favour for the transgenic traits, which i.e. will be the case for herbicide tolerance in the agro-ecosystem (Snow et al., 1999). In conclusion, given the right environment oilseed rape is apparently a potent donor of genes to *B. rapa* and introgressed plants survive and reproduce in the natural populations. Experiments in non-selective environments suggest that the fitness of some of the backcross plants will be equivalent to that of the wild parent. The reproductive fitness of interspecific F_1 hybrids can be even larger than that of both parents.

We have also shown that integration of genes in the plastid DNA or on C-chromosomes of oilseed rape will not provide safe guards towards natural introgression. However, the frequency of gene transfer can be reduced by efficient control of related weeds – i.e. by efficient herbicide control. Therefore the development of herbicide tolerant *B. rapa* will probably be slow in the conventionally managed fields and herbicide tolerant cultivars will provide easy and secure management of *B. rapa* over many years. As many farmers convert to agricultural practices with no or low herbicide usage weed problems may increase. The newly started organic production of oilseed rape may promote the presence of mixed weedy populations of oilseed rape and *B. rapa* in succeeding crops. Transgenes from neighboring GM oilseed rape may be transferred to *B. rapa* and *B. napus* in these populations. This scenario may gives rise to different transgenes becoming stacked in these reservoir populations and problems in relation to threshold values of GM in the organic production. Transfer of transgenes from oilseed rape to other wild relatives than *B. rapa* seems to be less likely.

Acknowledgement

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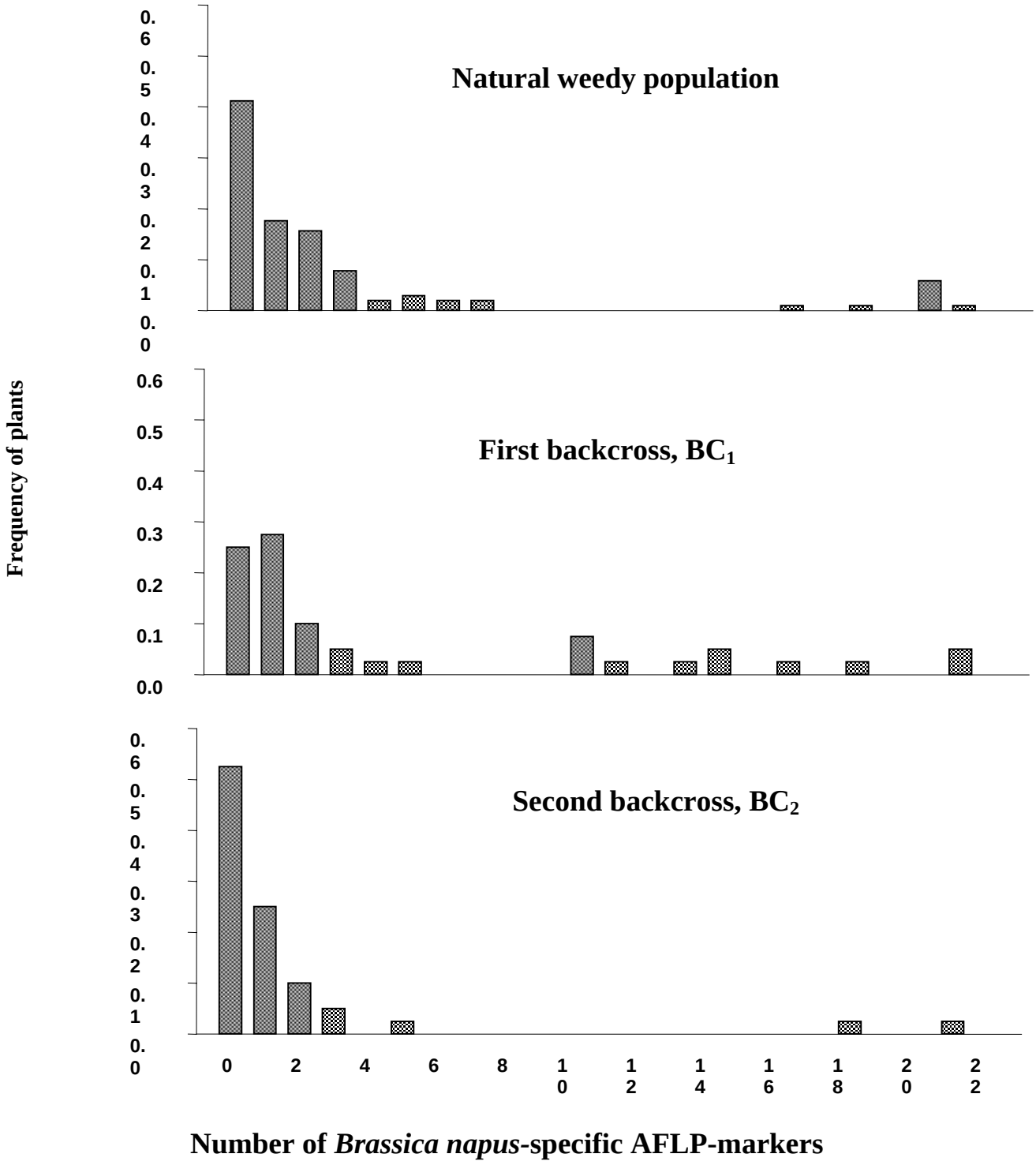

Figure 1. Distribution of oilseed rape specific AFLP markers in the weedy population of B. rapa *found together with oilseed rape in an organic field (top), in the BC_1 generation (middle) and BC_2 generation (bottom) from controlled crosses.*

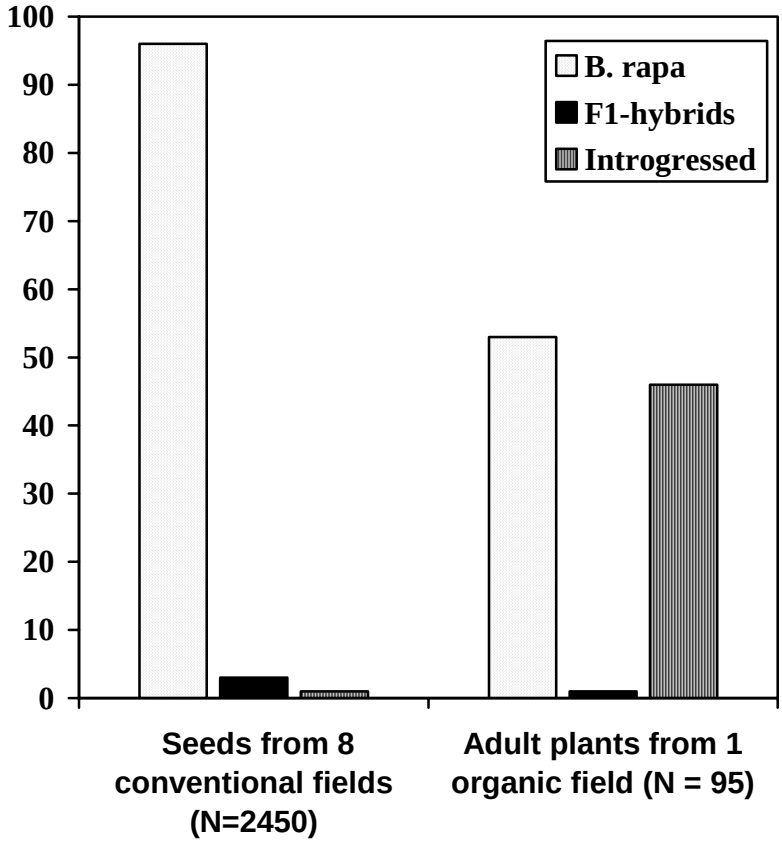

Figure 2. Frequency of B. rapa *and introgressed plants from mixed populations of oilseed rape and* B. rapa.

Paper V

Smarte biologiske løsninger begrænser spredning af transgener

(In: Tveit G., Madsen K.H. and Sandøe P. (eds.) "Vedr. bioteknologi og offentligheden, Rapport fra to forskningsprojekter om genmodificeret mad, planter og forsøgsdyr, Etik og Risikovurdering" (2003), s. 22-26, Center for Bioetik og Risikovurdering, København)

Smarte biologiske løsninger begrænser spredning af transgener.

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Hvis der skal dyrkes genetisk modificerede afgrøder, at det ønskeligt det foregår på en måde, der er skånsom for miljøet. Det skal desuden være muligt at praktisere på en ansvarlig måde i forhold til omkringliggende marker, således at enhver landmands frie ret til at vælge afgrøder og produktionsform er sikret, og uafhængig af hvad nabolandmanden vælger. Derfor er det vigtigt, at kende afgrøders evne til at sprede deres pollen og dermed transgener til andre marker med samme afgrøde eller til beslægtede vilde typer. Hvis en uønsket pollenspredning vil kunne finde sted, kan vi måske begrænse dens omfang – men hvordan?

Den første umiddelbare, om end noget naive, idé, der opstår, når man tænker på at begrænse pollenspredning kan være, at dyrkning af gensplejsede afgrøder kunne foregå i pollentætte drivhuse. Sådanne fysiske barrierer vil selvsagt have store økonomiske omkostninger både at etablere og opretholde, og vil kun komme på tale hvis en afgrøde med et meget stort økonomisk udbytte ser dagens lys. En anden mulighed kan være, at placere marker med den genetisk modificerede afgrøde i god afstand fra nabomarker og vilde typers voksesteder; et tiltag der kræver stor planlægning og mange ressourcer, og hvor pollenspredningens rækkevidde alligevel kan være svær at forudsige (Bock et al., 2002).

Biologisk indeslutning

En tredje type tiltag er at benytte biologiske barrierer for pollenspredningen. For eksempel er der udviklet sorter, der ikke producerer pollen (hansterile) – her er det plantens egne biologiske egenskaber, der udgør barrieren. Dette kan være ganske brugbart i nogle sammenhænge, men hvis plantens frø udgør det ønskede produkt, må en befrugtning finde sted. Det forudsætter tilstedeværelse af pollen, der så må komme fra en donor, der ikke er genetisk modificeret.

I planteceller findes den genetiske information i tre forskellige typer strukturer: Generne findes i kernen, i mitokondrier og i kloroplaster. Hos en lang række planter bliver mitokondrier og/eller kloroplaster ikke overført med pollen, men kun med frøet. Hvis et transgen derfor indsplejses i kloroplasterne, siges genet at være forsynet med en mekanisme til biologisk indeslutning. Afgrøder med transgener indsat i kloroplasterne kaldes trans-plastomiske.

Ved sameksistens mellem en transplastomisk afgrøde og beslægtede arter vil denne form for biologisk reduktion af spredningen begrænse transgenets udbredelse til marken, hvor afgrøden dyrkes, men de to arter kan stadig producere et afkom med transgenet, nemlig ved at den beslægtede art bestøver afgrøden. Hvis dette blandingsafkom (hybrid) dernæst bliver bestøvet af den beslægtede art igen, og dette gentager sig igennem flere generationer, vil der med tiden fås et afkom, der ligner den beslægtede art mere og mere, men stadig er transplastomisk. Den biologiske indeslutning er altså ikke perfekt. I undersøgelser af spredning af transgener har der været mest fokus på dannelsen af hybrider med de beslægtede arter som moderplanter, men med udgangspunkt i transplastomiske afgrøder er det interessant at vide i hvilket omfang, der dannes hybrider med afgrøden-arten som moderplante.

Raps som modelplante

Raps er oplagt at gensplejse, fordi den har et højt indhold af olie og protein i frøene, som anvendes til mange formål. For eksempel er genetisk modificeret raps med højere indhold af laurat i olien godkendt i Canada og USA (http://www.isaaa.org/kc/Bin/Global/index.htm). Olien sælges til industrien til anvendelse i fødevarer og kosmetik. En anden type genetisk modificeret raps, der er godkendt i Canada, har højere indhold af den umættede fedtsyre, oleinsyre, og olien anses derfor for mere ernæringsrigtig. Da raps er en afgrøde, hvis pollen transporteres langt med vinden og insekter, og den har beslægtede arter som den kan krydsbestøve med, vil det være oplagt at udvikle transplastomiske rapssorter for at begrænse pollenspredningen, hvilket da også er lykkedes for nylig (Hou et al., 2003).

Vi har udført et forsøg med en vinterraps sort (ikke-transplastomisk) og dens vilde slægtning agerkål, for at undersøge i hvilket omfang der dannes hybrider med raps som moderplante og agerkål som bestøver. Rapsen var ikke transplastomisk, da det ikke er en forudsætning for at bestemme hybridfrekvensen. Rapsen blev sammen med agerkål dyrket på et markareal i tre forskellige tætheder og i to forskellige forhold (tre raps til en agerkål og en raps til en agerkål), for at se hvordan forskellige konkurrenceforhold påvirker hybriddannelsen. Raps og agerkål er beslægtede arter, men har forskellig form og opbygning; raps er høj og kraftig, hvorimod agerkålen er mere spinkel. Derfor vil ændringer i plantesammensætningen og plantetætheden ændre planternes opvækstbetingelser og dermed også de to arters pollenproduktion.

Moderplanternes fitness/overlevelsesevne er desuden af betydning for hybriddannelsen. Hvis store og kraftige planter, som sætter mange frø, også producerer den største procentvise andel af hybridafkom, vil dette netto bidrage med flere hybrider, end hvis det er en spinkel plante med få frø og få hybridafkom. Frø fra en stor moderplante vil formentlig også i deres genetiske materiale have arvet evnen til at blive store og derved have bedre overlevelsesevne.

Hybridafkommet identificerede vi ved at lave et genetisk fingeraftryk – et DNA-fingerprint. Når man kender DNA-fingerprintet for raps og for agerkål, er det muligt at afgøre, om afkommet fra rapsen er fremkommet ved at rapsen har bestøvet sig selv, eller om der er sket krydsbestøvning med agerkål. Hvis der er agerkål-markører tilstede, er agerkålen faderen. Vi har undersøgt 50 individer fra 10 moderplanter fra hver af de seks plottyper, i alt 3000 individer.

Genspredning fra transplastomisk raps

I Tabel 1 fremgår hybriddannelsen i procent indenfor hver plottype. Starter vi med at se på tabellen kolonnevis ser det ved *lav* plantetæthed ud til at være uden betydning om der er en *høj* eller *lav* forekomst af agerkål i populationen; bidraget til pollenskyen ser ud til at være den samme. Ser vi på resultaterne ved *mellem* og *høj* tæthed dannes der, ikke overraskende, færre hybrider med agerkål som far, når forekomsten af agerkål i plottene er lavest, altså i 3:1 plottene. Ved disse tætheder har den lavere forekomst af agerkål i populationen altså en effekt på pollenskyen. Ser vi nu på tabellen rækkevis ser det i de plot hvor raps og agerkål er lige repræsenteret (1:1) ikke ud til at plantetætheden har en effekt på hvor hyppigt episoden indtræffer. Plantetætheden har derimod en effekt, når agerkål er sjældnere repræsenteret (3:1); ved *mellem* og *høj* plantetæthed dannes der færre hybrider, hvilket formentlig skyldes at agerkålen bliver klemt.

Konklusionen baseret på disse resultater er altså i overensstemmelse med, hvad man umiddelbart ville forvente, nemlig, at hvis der dyrkes raps ved *mellem* til *høj* plantetæthed, og man sørger for, at der er så få agerkål i marken som muligt, vil der dannes færrest hybrider. Altså må rådet til landmanden være, at rapsmarken skal være tæt og agerkål bekæmpes.

Tabel 1. Den gennemsnitlige procentvise forekomst af hybridafkom pr. raps moderplante (± 95% konfidensinterval)

	Plantetætheder			
Forhold (raps:agerkål)	Lav (16 planter pr. m^2)	Mellem (44 planter pr. m^2)	Høj (100 planter pr. m^2)	Alle Tætheder
3:1	2,6 % (±1,4)	0,4 % (±0,6)	1 % (±0,9)	1,3 % (±0,6)
1:1	3 % (±1,5)	2,8 % (±1,4)	4 % (±1,7)	3,3 % (±0,9)
Alle forhold	2,8 % (±1,4)	1,6 % (±1,1)	2,5 % (±1,4)	2,3 % (±0,5)

Hvad betyder disse procenter omsat i praksis? I Tabel 2 har vi korrigeret den gennemsnitlige forekomst af hybrider for antallet af frø produceret af de enkelte moderplanter, således, at det faktiske antal hybrider fremgår. (Det er jo af stor betydning om de planter, der producerer mange hybrider, sætter få eller mange frø). Heraf ses det, at en *høj* plantetæthed generelt bevirker at der produceres få hybrider, og at en *lav* plantetæthed generelt bevirker at der produceres relativt mange. Det at tendensen er generel viser at hybridproduktionen tilsyneladende er uafhængig af artssammensætningen (3:1 og 1:1) ved disse tætheder. Derfor ville der ved *mellem* tæthed og begge forhold forventes en hybridproduktion på et niveau mellem denne *lave* og den *høje* tæthed, hvilket da omtrent også er tilfældet ved forholdet 1:1. Derimod bliver hybridproduktion faktisk den laveste observerede overhovedet i forsøget når der er en *mellem* tæthed og et forholdet på 3:1. En forklaring kan være, at netop denne kombination af tæthed (*mellem*) og forhold (3:1) måske er særlig favorabel for raps, og derfor gør det særlig svært for agerkål at klare sig i konkurrencen. Den større konkurrence fra rapsen bevirker måske, at agerkålen producerer mindre pollen, således at

dens bidrag til pollenskyen bliver mindre, og dermed muligheden for at der produceres hybrider på raps.

Tabel 2. Det gennemsnitlige antal hybridafkom pr. raps moderplante (± 95% konfidensinterval)

	Tætheder			
Forhold (raps:agerkål)	Lav (16 planter pr. m^2)	Mellem (44 planter pr. m^2)	Høj (100 planter pr. m^2)	Alle tætheder
3:1	116 (±71)	7 (±9)	14 (±15)	46 (±30)
1:1	118 (±87)	62 (±65)	19 (±11)	66 (±38)
Alle forhold	133 (±58)	35 (±35)	16 (±9)	56 (±24)

Genspredning fra "ikke-indesluttet" raps

Udover at det er interessant at se, hvad den faktiske produktion af hybrider vil være, hvis der dyrkes transplastomisk raps, er det interessant at se, hvad der undgås ved det. Hvor villigt produceres der hybrider med agerkål som mor? I Tabel 3 ses den gennemsnitlige procentvise forekomst af hybrider produceret på agerkål (Pertl et al., 2002). I dette markforsøg var der udover raps og agerkål også inkluderet deres hybrid. Idet hybriderne var fader i meget få tilfælde (1,5% ved lav tæthed og 3:1:1 forholdet og 0 % ved alle andre forhold og tætheder), mener vi det er rimeligt at sammenligne dette forsøg med vores resultater, selvom vi ikke har hybrider med i forsøget. Forsøgene adskiller sig også ved at vi benyttede en vinterrapssort, hvorimod Pertl et al. (2002) benyttede en vårrapssort. Vinterraps er mere konkurrencedygtig, og giver er større udbytte end vårraps, hvorfor hybrider produceret på vinterraps moderplanter antagelig har bedre overlevelsesevne og dermed et større potentiale for at videreføre transgener til vilde slægtninge. Derudover dyrkes vinterraps hyppigere end vårraps og er derfor mere relevant at undersøge med hensyn til spredning af transgener.

Tabel 3. Den gennemsnitlige procentvise forekomst af hybridafkom pr. agerkål moderplante (Pertl et al., 2002)

	Plantetætheder	
Forhold (raps:agerkål:hybrid)	Lav (16 planter pr. m^2)	Høj (100 planter pr. m^2)
35:**1**:0	51 %	1,3 %
3:**1**:1	3,7 %	0,4 %
1:**1**:3	0 %	0 %

Produktionen af hybrider ved 3:1 (**3:1**:1) forholdet og lav tæthed var 2,6 % med raps som moderplante og 3,7 % med agerkål som moderplante, den har altså været ca. 1/3 højere med agerkål som moderplante.. Ved 3:1 (**3:1**:1) forholdet og høj tæthed var den 1 % og 0,4 % med raps og agerkål som moderplante. Hyppigheden var altså dobbelt så høj når raps var moderplante. Ved 1:1 (**1:1**:3) forholdet fandt vi at hybridproduktionen var 3 % (lav tæthed) og 4 % (høj tæthed) hvis raps var moderplante, hvorimod den var 0 % ved begge tætheder hvis agerkål var moderplante. Det vil sige at genspredningen ikke er blevet reduceret under disse forhold. Hvor hybriderne produceres og hvor mange, er således afhængigt af artssammensætningen og tætheden. I alt produceres flest ved 3:1 forholdet og *lav* tæthed (6,3%, antallet af hybrider produceret på raps plus agerkål) og færrest ved 3:1 forholdet og *høj* tæthed (1,4%, antallet af hybrider produceret på raps plus agerkål). De sidstnævnte dyrkningsbetingelser er altså dem, der bør vælges, hvis der skal dyrkes gensplejset raps, som er genetisk modificeret på traditionel vis, fordi den samlede hybridproduktion derved bliver lavest. Vi kender ikke produktionen af hybrider på agerkål ved *mellem* tæthed, men selvom den er høj er det underordnet, hvis der dyrkes transplastomisk raps, fordi hybriderne da ikke får overført transgenet med pollen. Derfor, da det er ved mellem til høj tæthed og forholdet 3:1 der produceres færrest hybrider på raps er det ved disse betingelser, der bør dyrkes transplastomisk raps.

Konklusioner

Raps som moderplante bidrager altså stadig til den samlede hybridproduktion, og den vil være bestående ved dyrkning af transplastomisk raps, såvel som ved dyrkning af raps, der er genetisk modificeret på traditionel vis. Men man kan sige at hybridproduktionen er mere kontrolleret, idet alle hybrider findes inden for den mark, hvor den genetisk modificerede raps dyrkes. Denne kontrol med afkommet er måske kun midlertidig, da der under transporten af frøene efter høst, kan forekomme et vist frøspild, men denne situation adskiller sig ikke fra den mekaniske spredningsrisiko, der er ved dyrkning af traditionelt modificeret raps.

I dette forsøg har vi set på hybriddannelsen. Det næste trin i spredningen af transgener fra raps til agerkål afhænger af hybridernes overlevelsesevne, samt hyppigheden hvormed de bestøves af agerkål igen eller selvbestøver. Vi har derfor gennemført et markforsøg, hvor vi har ladet hybriderne sameksistere med raps og agerkål i forskellige forhold og i samme tætheder som vi tidligere har benyttet. Vi er på nuværende tidspunkt i gang med en faderskabsanalyse.

Vi har analyseret hybriddannelsen mellem raps og agerkål, når raps fungerer som mor. Transplastomisk raps kan være medvirkende til at nedsætte denne hybriddannelse, og dermed eventuelle risici forbundet hermed. En anden klar fordel ved dyrkning af transplastomisk raps er, at man undgår krydsbestøvning mellem marker med samme type afgrøde. Selvom vinden blæser pollen mellem markerne eller bierne flyver imellem dem, vil pollenet ikke indeholde transgener.

Litteratur

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Paper VI

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Declarations by co-authors

Mission

To promote an innovative and environmentally sustainable technological development within the areas of energy, industrial technology and bioproduction through research, innovation and advisory services.

Vision

Risø's research **shall extend the boundaries** for the understanding of nature's processes and interactions right down to the molecular nanoscale.

The results obtained shall **set new trends** for the development of sustainable technologies within the fields of energy, industrial technology and biotechnology.

The efforts made **shall benefit** Danish society and lead to the development of new multi-billion industries.